METHODS FOR MODULATING AN IMMUNE RESPONSE BY MODULATING KRC ACTIVITY

Related Applications

This application is a National Stage of PCT/US2004/036641, filed November 3, 2004, which is a continuation-in-part of U.S. application No. 10/701,401, filed November 3, 2003, which claims the benefit of priority to PCT application PCT/US02/14166, filed May 3, 2002, which claims the benefit of U.S. Provisional Application Serial No. 60/288,369, filed May 3, 2001. The entire contents of each of these applications are incorporated herein by this reference.

Background of the Invention

Transcription factors are a group of molecules within the cell that function to connect the pathways from extracellular signals to intracellular responses. Immediately after an environmental stimulus, these proteins which reside predominantly in the cytosol are translocated to the nucleus where they bind to specific DNA sequences in the promoter elements of target genes and activate the transcription of these target genes. One family of transcription factors, the ZAS (zinc finger-acidic domain structures) DNA binding protein family is involved in the regulation of gene transcription, DNA recombination, and signal transduction (Mak, C.H., *et al.* 1998. *Immunogenetics* 48: 32-39).

Zinc finger proteins are identified by the presence of highly conserved Cys2His2 zinc fingers (Mak, C.H., *et al.* 1998. *Immunogenetics* 48: 32-39). The zinc fingers are an integral part of the DNA binding structure called the ZAS domain. The ZAS domain is comprised of a pair of zinc fingers, a glutamic acid/aspartic acid-rich acidic sequence and a serine/threonine rich sequence (Mak, C.H., *et al.* 1998. *Immunogenetics* 48: 32-39). The ZAS domains have been shown to interact with the kB like *cis*-acting regulatory elements found in the promoter or enhancer regions of genes. The ZAS proteins recognize nuclear factor kB binding sites which are present in the enhancer sequences of many genes, especially those involved in immune responses (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648). The ZAS DNA binding proteins have been

shown to be transcription regulators of these target genes (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648; Wu *et al.* 1998. *Science 281*, 998-1001).

The zinc finger transcription factor Kappa Recognition Component ("KRC") is a member of the ZAS DNA binding family of proteins (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648; Wu *et al.* 1998. *Science 281*, 998-1001). The KRC gene was identified as a DNA binding protein for the heptameric consensus signal sequences involved in somatic V(D)J recombination of the immune receptor genes (Mak, C. H., *et al.* 1994. *Nuc.Acid Res. 22:* 383-390). KRC is a substrate for epidermal growth factor receptor kinase and p34cdc2 kinase in vitro (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648). However, other functions of KRC and the signal transduction pathways that activate KRC in vivo were not known.

Gene-specific transcription factors provide a promising class of targets for novel therapeutics because they provide substantial specificity and are known to be involved in human disease. A number of extremely effective presently marketed drugs act, at least indirectly, by modulating gene transcription. For instance, in many cases of heart disease, the LDL receptor is pathogenically down-regulated at the level of transcription by intracellular sterol levels. The drug compactin, an inhibitor of HMG CoA reductase, functions by up-regulating transcription of the LDL receptor gene which leads to clearance of cholesterol from the blood stream.

In another example, transcription factors can be modulated to regulate an immune response. In autoimmune diseases, self-tolerance is lost and the immune system attacks "self" tissue as if it were a foreign target. Many autoimmune diseases are presently known, such as multiple sclerosis (MS), rheumatoid arthritis, insulin-dependent diabetes mellitus, hemolytic anemias, rheumatic fever, Crohn's disease, Guillain-Barre syndrome, psoriasis, glomerulonephritis, autoimmune hepatitis, multiple sclerosis, etc. In diseases such as these, inhibiting the immune response is desirable. In addition, inhibiting the body's immune response is beneficial in prevention, for example, of organ transplant rejection. Conversely, enhancing the immune response is beneficial in certain circumstances such as the treatment of AIDS, cancer, atherosclerosis and diabetic complications (Sen, P. *et al.* 1996. *FASEB Journal* 10:709-720, 1996). Urgently needed are efficient methods of identifying pharmacological agents or drugs which are active at the level of gene transcription. Specifically, agents for use

modulating such cellular processes in T cells are needed to regulate the immune response. Agents and methods of using such agents in modulation of cell survival, proliferation, differentiation and/or motility would be of great benefit.

Summary of the Invention

The present invention is based, at least in part, on the discovery that KRC molecules have multiple important functions as modulating agents in regulating a wide variety of cellular processes. The invention is based, at least in part, on the discovery that KRC inhibits NFkB transactivation, increases TNF-alpha induced apoptosis, inhibits JNK activation, inhibits endogenous TNF-alpha expression, promotes immune cell proliferation and immune cell activation (*e.g.*, in T cells (such as Th1 and/or Th2 cells), B cells, or macrophages), activates IL-2 expression *e.g.*, by activating the AP-1 transcription factor, and increases actin polymerization. The present invention also demonstrates that KRC interacts with TRAF. Furthermore, the present invention demonstrates that KRC physically interacts with the c-Jun component of AP-1 to control its degradation. The present invention also demonstrates that KRC is downstream of several lymphocyte membrane receptors, including TNFR, TCR and TGFβR. Upon TNFR signaling, KRC associates with the adaptor protein TRAF2 to inhibit NFκB and JNK-dependent gene expression. Upon TCR stimulation, KRC expression is rapidly induced and KRC physically associates with the c-Jun transcription factor to augment AP-1 dependent gene transcription. KRC knock-out (KO) T cells have impaired production of AP-1-dependent genes such as CD69 and IL-2. Upon TCR stimulation KRC also associates with the Th2-specific transcription factor GATA3, and T cells lacking KRC have impaired production of GATA3 dependent Th2 cytokines, IL-4, IL-5 and IL-13. Finally, upon TGFβ receptor signaling, KRC physically associates with the transcription factor SMAD3 to activate IgA germline transcription in B cells, since KRC KO B cells have impaired IgA production and germline Igα (GLα) gene transcription.

In one aspect, the invention pertains to a method for identifying a compound which modulates an interaction between a first and a second polypeptide comprising: (a) contacting a cell having a first polypeptide comprising a binding portion of a KRC polypeptide and a second polypeptide comprising a binding portion of a polypeptide selected from the group consisting of: Jun, GATA3, SMAD, or Runx2 in the presence and the absence of a test compound; and (b) determining the degree of interaction

between the first and the second polypeptide in the presence and the absence of the test compound, to thereby identify a compound which modulates an interaction between a first and a second polypeptide.

In one embodiment, the first polypeptide comprises at least one KRC zinc finger domain. In one embodiment, the second polypeptide is a c-Jun polypeptide. In another embodiment, the second polypeptide is a SMAD2 polypeptide. In another embodiment, the second polypeptide is a SMAD3 polypeptide.

In one embodiment, the first polypeptide is derived from an exogenous source. In another embodiment, the second polypeptide is derived from an exogenous source.

In one embodiment, the cell is a yeast cell.

In one embodiment, determining the ability of the test compound to modulate the interaction of the first polypeptide and the second polypeptide comprises determining the ability of the compound to modulate growth of the yeast cell on nutritionally selective media.

In another embodiment, determining the ability of the test compound to modulate the interaction of the first polypeptide and the second polypeptide comprises determining the ability of the compound to modulate expression of a reporter gene in the yeast cell.

In one embodiment, determining the ability of the test compound to modulate the interaction of the first polypeptide and the second polypeptide comprises determining the ability of the test compound to modulate the coimmunoprecipitation of the first polypeptide and the second polypeptide.

In another embodiment, determining the ability of the test compound to modulate the interaction of the first polypeptide and the second polypeptide comprises determining the ability of the test compound to modulate signaling via a signal transduction pathway involving KRC in the cell.

In one embodiment, at least one of TNFα production, IL-2 production, AP-1 activity, Ras and Rac activity, actin polymerization, ubiquitination of AP-1, ubiquitination of TRAF, ubiquitination of Runx2, degradation of c-Jun, degradation of c-Fos degradation of SMAD, degradation of Runx2, degradation of GATA3, GATA3 expression, Th2 cell differentiation, Th2 cytokine production, IgA production, GLα

transcription (Igα chain germline transcription), and/or osteocalcin gene transcription is measured.

In one embodiment, ubiquitination or degradation of c-fos, c-Jun, SMAD3, GATA3 or Runx2 is measured.

In one embodiment, AP-1, TRAF2 or Runx2 ubiquitination is measured.

In one embodiment, the binding of first and second polypeptide is inhibited.

In one embodiment, the binding of first and second polypeptide is stimulated.

In another aspect, the invention pertains to a method of identifying a compound that modulates a mammalian KRC biological activity comprising:

(a) contacting cells deficient in KRC or a molecule in a signaling pathway involving KRC with a test compound; and

(b) determining the effect of the test compound on the KRC biological activity, the test compound being identified as a modulator of the biological activity based on the ability of the test compound to modulate the biological activity in the cells deficient in KRC or a molecule in a signaling pathway involving KRC to thereby identify a compound that modulates a mammalian KRC biological activity.

In one embodiment, the biological activity of KRC is selected from the group consisting of modulation of: modulation of a TGFβ signaling pathway, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD, modulation of degradation of Runx, modulation of degradation of GATA3, modulation of GATA3 expression, modulation of Th2 cell differentiation, modulation of Th2 cytokine production, modulation of IgA production, modulation of GLα transcription, or modulation of osteocalcin gene transcription.

In one embodiment, the cells are in a non-human animal deficient in KRC or a molecule in a signal transduction pathway involving KRC and the cells are contacted with the test compound by administering the test compound to the animal.

In one aspect, the invention pertains to a method of identifying compounds useful in modulating a biological activity of mammalian KRC comprising, a) providing an indicator composition comprising mammalian KRC or a molecule in a signal transduction pathway involving KRC; b) contacting the indicator composition with each

member of a library of test compounds; c) selecting from the library of test compounds a compound of interest that modulates a biological activity of KRC or the molecule in a signal transduction pathway involving KRC; to thereby identify a compound that modulates a biological activity of mammalian KRC, wherein the biological activity of KRC is selected from the group consisting of: modulation of a TGFβ signaling pathway, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD, modulation of degradation of Runx, modulation of degradation of GATA3, modulation of GATA3 expression, modulation of Th2 cell differentiation, modulation of Th2 cytokine production, modulation of IgA production, modulation of GLα transcription, and modulation of osteocalcin gene transcription.

In one embodiment, the indicator composition is a cell that expresses KRC, and at least one molecule selected from the group consisting of: c-Jun, c-Fos, AP-1, GATA3, SMAD, and Runx2 protein.

In one embodiment, the indicator composition is a cell free composition.

In another embodiment, the invention pertains to a non-human animal, in which the gene encoding the KRC gene is misexpressed.

In one embodiment, the animal is a transgenic animal.

In one embodiment, the transgenic animal is a mouse.

In one embodiment, the KRC gene is disrupted by removal of DNA encoding all or part of the KRC protein.

In one embodiment, the animal is homozygous for the disrupted gene.

In one embodiment, the animal is heterozygous for the disrupted gene.

In one embodiment, the animal is a transgenic mouse with a transgenic disruption of the KRC gene.

In one embodiment, the disruption is an insertion or deletion.

In one aspect, the invention pertains to a transgenic mouse comprising in its genome an exogenous DNA molecule that functionally disrupts a KRC gene of said mouse, wherein said mouse exhibits a phenotype characterized by impaired Th2 cell development, decreased Th2 cytokine production, impaired TGFβR signaling in B cells,

decreased IgA secretion and decreased transcription of the GLα gene, relative to a wildtype mouse.

Detailed Description of the Invention

The present invention is based, at least in part, on the discovery that KRC molecules regulate a wide variety of cellular processes, including inhibiting NFkB transactivation, increasing TNF-alpha induced apoptosis, inhibiting JNK activation, inhibiting endogenous TNF-alpha expression, activating immune cell proliferation and immune cell activation (*e.g.*, in Th1 cells), activating IL-2 expression *e.g.*, by activating the AP-1 transcription factor, and increasing actin polymerization.

The present invention also demonstrates that that KRC interacts with TRAF molecules. The interaction between KRC and TRAF involves the C domain of TRAF and amino acid residues 204 to 1055 of KRC. Furthermore, the present invention demonstrates that KRC physically interacts with the c-Jun component of AP-1 to control its degradation. KRC also interacts with GATA3, SMAD, *e.g.*, SMAD2 and SMAD3, and Runx2 to control their degradation, and ubiquitinates TRAF and Runx2.

Furthermore, the present invention demonstrates upon TCR stimulation KRC also associates with the Th2-specific transcription factor GATA3, and T cells lacking KRC have impaired production of GATA3 dependent Th2 cytokines, such as, IL-4, IL-5 and IL-13. In addtion, upon TGFβ receptor signaling, KRC physically associates with members of the SMAD transcription factor family, *e.g.*, SMAD2 and SMAD3, to activate IgA germline transcription in B cells.

The KRC protein (for κB binding and putative recognition component of the V(D)J Rss), referred to interchangeably herein as *Schnurri-3* (Shn3), is a DNA binding protein comprised of 2282 amino acids. KRC has been found to be present in T cells, B cells, and macrophages. The KRC cDNA sequence is set forth in SEQ ID NO:1. The amino acid sequence of KRC is set forth in SEQ ID NO:2. KRC is a member of a family of zinc finger proteins that bind to the kB motif (Bachmeyer, C, *et al.*, 1999. *Nuc. Acids. Res.* 27(2):643-648). Zinc finger proteins are divided into three classes represented by KRC and the two MHC Class I gene enhancer binding proteins, MBP1 and MBP2 (Bachmeyer, C, *et al.*, 1999. *Nuc. Acids. Res.* 27(2):643-648).
Zinc finger proteins are identified by the presence of highly conserved Cys2His2 zinc fingers. The zinc fingers are an integral part of the DNA binding structure called the

ZAS domain. The ZAS domain is comprised of a pair of zinc fingers, a glutamic acid/aspartic acid-rich acidic sequence and a serine/threonine rich sequence. The ZAS domains have been shown to interact with the kB like *cis*-acting regulatory elements found in the promoter or enhancer regions of genes. The genes targeted by these zinc finger proteins are mainly involved in immune responses.

The KRC ZAS domain, in particular, has a pair of Cys2-His2 zinc fingers followed by a glutamic acid/aspartic acid-rich acidic sequence and five copies of the serine/threonine-proline-X-arginine/lysine sequence. Southwestern blotting experiments, electrophoretic mobility shift assays (EMSA) and methylation interference analysis has also demonstrated that KRC recombinant proteins bind to the κB motif as well as to the Rss sequence (Bachmeyer, *et al.* 1999. *Nuc. Acid Res. 27*, 643-648; Wu *et al.* 1998. *Science 281*, 998-1001) and do so in highly ordered complexes (Mak, C. H., *et al.* 1994. *Nuc.Acid Res. 22*, 383-390.; Wu *et al.* 1998. *Science 281*, 998-1001).

Similar zinc finger-acidic domain structures are present in human KBP1, MBP1 and MBP2, rat ATBP1 and ATBP2, and mouse αA-CRYBP proteins. KRC has recently been shown to regulate transcription of the mouse metastasis-associated gene, *s100A4/mts1**, by binding to the Sb element (a kB like sequence) of the gene. (Hjelmsoe, I., *et al.* 2000. *J. Biol. Chem.* 275(2): 913-920). KRC is regulated by post-translational modification as evidenced by the fact that pre-B cell nuclear protein kinases phosphorylate KRC proteins on serine and tyrosine residues. Phosphorylation increases DNA binding, providing a mechanism by which KRC may respond to signals transmitted from the cell surface (Bachmeyer, C, *et al.*, 1999. *Nuc. Acids. Res.* 27(2):643-648). Two prominent ser/thr-specific protein kinases that play a central role in signal transduction are cyclic AMP-dependent protein kinase A (PKA) and the protein kinase C (PKC family). Numerous other serine/threonine specific kinases, including the family of mitogen-activated protein (MAP) kinases serve as important signal transduction proteins which are activated in either growth-factor receptor or cytokine receptor signaling. Other protein ser/thr kinases important for intracellular signaling are Calcium-dependent protein kinase (CaM-kinase II) and the c-raf-protooncogene. KRC is known to be a substrate for epidermal growth factor receptor kinase and p34cdc2 kinase *in vitro*.

The results of a yeast two hybrid screen using amino acid residues 204 to 1055 of KRC (which includes the third zinc finger) as bait demonstrate that KRC interacts with the TRAF family of proteins and that this interaction occurs through the TRAF C domain and that KRC interacts with higher affinity with TRAF2 than with TRAF5 and TRAF6. (See Example 1).

Recent research has lead to the isolation of polypeptide factors named TRAFs for tumor necrosis factor receptor associated factors, which participate in the TNFR signal transduction cascade. Six members of the TRAF family of proteins have been identified in mammalian cells (reviewed in Arch, R.H., *et al.* 1998. *Genes Dev. 12*, 2821-2830). All TRAF proteins, with the exception of TRAF1, contain an amino terminal RING finger domain with a characteristic pattern of cysteines and histidines that coordinate the binding of Zn2+ ions (Borden, K. L. B., *et al.* 1995. *EMBO J 14*, 1532-1521), which is followed by a stretch of multiple zinc fingers. All TRAFs share a highly conserved carboxy-terminal domain (TRAF-C domain) which is required for receptor binding and can be divided into two parts, a highly conserved domain which mediates homo and heterodimerization of TRAF proteins and also the association of the adapter proteins with their associated receptors and an amino-terminal half that displays a coiled-coil configuration. TRAF molecules have distinct patterns of tissue distribution, are recruited by different cell surface receptors and have distinct functions as revealed most clearly by the analysis of TRAF-deficient mice (*see* Lomaga, M. A., *et al.* 1999. *Genes Dev. 13*, 1015-24; Nakano, H., *et al.* 1999. *Proc. Natl. Acad. Sci. USA 96*, 9803-9808; Nguyen, L. T., *et al.* 1999. *Immunity 11*, 379-389; Xu, Y., *et al.* 1996. *Immunity 5*, 407-415.; Yeh, W. C., *et al.* 1997. *Immunity 7*, 715-725).

Tumor necrosis factor (TNF) is a cytokine produced mainly by activated macrophages which elicits a wide range of biological effects. These include an important role in endotoxic shock and in inflammatory, immunoregulatory, proliferative, cytotoxic, and anti-viral activities (reviewed by Goeddel, D. V. *et al.*, 1986. *Cold Spring Harbor Symposia on Quantitative Biology* 51: 597-609; Beutler, B. and Cerami, A., 1988. *Ann. Rev. Biochem.* 57: 505-518; Old, L. J., 1988. *Sci. Am.* 258(5): 59-75; Fiers, W. 1999. *FEBS Lett.* 285(2):199-212). The induction of the various cellular responses mediated by TNF is initiated by its interaction with two distinct cell surface receptors, an approximately 55 kDa receptor termed TNFR1 and an approximately 75 kDa receptor

termed TNFR2. Human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher, H. *et al.*, 1990. *Cell* 61:351; Schall, T. J. *et al.*, 1990. *Cell* 61: 361; Smith, C. A. *et al.*, 1990 *Science* 248: 1019; Lewis, M. *et al.*, 1991. *Proc. Natl. Acad. Sci. USA* 88: 2830-2834; Goodwin, R. G. *et al.*, 1991. *Mol. Cell. Biol.* 11:3020-3026).

TNFα binds to two distinct receptors, TNFR1 and TNFR2, but in most cell types NFκB activation and JNK/SAPK activation occur primarily through TNFR1. TNFR1 is known to interact with TRADD which functions as an adaptor protein for the recruitment of other proteins including RIP, a serine threonine kinase, and TRAF2. Of the six known TRAFs, TRAF2, TRAF5 and TRAF6 have all been linked to NFκB activation (Cao, Z., *et al.* 1996. *Nature 383:* 443-6; Rothe, M., *et al.* 1994. *Cell 78:* 681-692; Nakano, H., *et al.* 1996. *J. Biol. Chem. 271*:14661-14664), and TRAF2 in particular has been linked to activation of the JNK/SAPK proteins as shown unequivocally by the failure of TNFα to activate this MAP kinase in cells lacking TRAF2 or expressing a dominant negative form of TRAF2 (Yeh, W. C., *et al.* 1997. *Immunity 7*: 715-725; Lee, S. Y., *et al.* 1997. *Immunity 7:*1-20).

Various aspects of the invention are described in further detail in the following subsections:

I. Definitions

As used herein, the term "KRC" , used interchangeably with "Shn3" refers to κB binding and putative recognition component of the V(D)J Rss. The nucleotide sequence of KRC is set forth in SEQ ID NO:1 and the amino acid sequence of KRC is set forth in SEQ ID NO:2. The amino acid sequence of the ZAS domain of KRC is set forth in amino acids 1497-2282 of SEQ ID NO:2 (SEQ ID NO:8). The amino acid sequence of KRC tr is shown in amino acid residues 204 to 1055 of SEQ ID NO:2. As used herein, the term "KRC", unless specifically used to refer a specific SEQ ID NO, will be understood to refer to a KRC family polypeptide as defined below.

"KRC family polypeptide" is intended to include proteins or nucleic acid molecules having a KRC structural domain or motif and having sufficient amino acid or nucleotide sequence identity with a KRC molecule as defined herein. Such family members can be naturally or non-naturally occurring and can be from the same or different species. For example, a family can contain a first protein of human origin, as

well as other, distinct proteins of human origin or, alternatively, can contain homologues of non-human origin. Preferred members of a family may also have common functional characteristics. Preferred KRC polypeptides comprise one or more of the following KRC characteristics: a pair of Cys2-His2 zinc fingers followed by a Glu- and Asp-rich acidic domain and five copies of the ser/Thr-Pro-X-Arg/Lys sequence thought to bind DNA.

As used herein, the term "KRC activity", "KRC biological activity" or "activity of a KRC polypeptide" includes the ability to modulate an activity regulated by KRC or a signal transduction pathway involving KRC. For example, in one embodiment a KRC biological activity includes modulation of an immune response. Exemplary KRC activities include *e.g.*, modulating: immune cell activation and/or proliferation (such as by modulating cytokine gene expression), cell survival (*e.g.*, by modulating apoptosis), signal transduction via a signaling pathway (*e.g.*, an NFkB signaling pathway, a JNK signaling pathway, and/or a TGFβ signaling pathway), actin polymerization, ubiquitination of AP-1, ubiquitination of TRAF, ubiquitination of Runx2, degradation of c-Jun, degradation of c-Fos, degradation of SMAD, degradation of Runx 2, degradation of GATA3, GATA3 expression, Th2 cell differentiation, Th2 cytokine production, IgA production, GLα transcription, and/or osteocalcin gene transcription.

As used herein, the various forms of the term "modulate" are intended to include stimulation (*e.g.*, increasing or upregulating a particular response or activity) and inhibition (*e.g.*, decreasing or downregulating a particular response or activity).

As described in the appended Examples, KRC increases immune cell activation and cytokine production. In addition, when KRC is overexpressed, it results in the inhibition of NFkB and JNK signaling pathways. Inhibition of these pathways is associated with cellular inflammatory and apoptotic responses. In one embodiment, the KRC activity is a direct activity, such as an association with a KRC-target molecule or binding partner. As used herein, a "target molecule", "binding partner" or "KRC binding partner" is a molecule with which a KRC protein binds or interacts in nature, such that KRC mediated function is achieved.

As used herein the term "TRAF" refers to TNF Receptor Associated Factor (See *e.g.*, Wajant et al, 1999, *Cytokine Growth Factor Rev* 10:15-26). The "TRAF" family includes a family of cytoplasmic adapter proteins that mediate signal transduction from

many members of the TNF-receptor superfamily and the interleukin-1 receptor (see *e.g.*, Arch, R.H. *et al.*, 1998, *Genes Dev.* 12:2821-2830). As used herein, the term "TRAF C domain" refers to the highly conserved sequence motif found in TRAF family members.

As used herein, the terms "TRAF interacting portion of a KRC molecule" or "c-Jun interacting portion of a KRC molecule" includes a region of KRC that interacts with TRAF or c-Jun. In a preferred embodiment, a region of KRC that interacts with TRAF or c-Jun is amino acid residues 204-1055 of SEQ ID NO:2 (SEQ ID NO:7). As used herein, the term "KRC interacting portion of a TRAF molecule" or "KRC interacting portion of a TRAF molecule" includes a region of TRAF or c-Jun that interacts with KRC. In a preferred embodiment, a region of TRAF that interacts with KRC is the TRAF C domain.

The term "interact" as used herein is meant to include detectable interactions between molecules, such as can be detected using, for example, a yeast two hybrid assay or coimmunoprecipitation. The term interact is also meant to include "binding" interactions between molecules. Interactions may be protein-protein or protein-nucleic acid in nature.

As used herein, the term "contacting" (*i.e.*, contacting a cell *e.g.* an immune cell, with an compound) is intended to include incubating the compound and the cell together *in vitro* (*e.g.*, adding the compound to cells in culture) or administering the compound to a subject such that the compound and cells of the subject are contacted *in vivo*. The term "contacting" is not intended to include exposure of cells to a KRC modulator that may occur naturally in a subject (*i.e.*, exposure that may occur as a result of a natural physiological process).

As used herein, the term "test compound" includes a compound that has not previously been identified as, or recognized to be, a modulator of KRC activity and/or expression and/or a modulator of cell growth, survival, differentiation and/or migration.

The term "library of test compounds" is intended to refer to a panel comprising a multiplicity of test compounds.

As used herein, the term "cell free composition" refers to an isolated composition which does not contain intact cells. Examples of cell free compositions include cell extracts and compositions containing isolated proteins.

As used herein, an "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid.

In one embodiment, nucleic acid molecule of the invention is an siRNA molecule. In one embodiment, a nucleic acid molecule of the invention mediates RNAi. RNA interference (RNAi) is a post-transcriptional, targeted gene-silencing technique that uses double-stranded RNA (dsRNA) to degrade messenger RNA (mRNA) containing the same sequence as the dsRNA (Sharp, P.A. and Zamore, P.D. 287, 2431-2432 (2000); Zamore, P.D., *et al. Cell* 101, 25-33 (2000). Tuschl, T. *et al. Genes Dev.* 13, 3191-3197 (1999); Cottrell TR, and Doering TL. 2003. Trends Microbiol. 11:37-43; Bushman F.2003. Mol Therapy. 7:9-10; McManus MT and Sharp PA. 2002. Nat Rev Genet. 3:737-47). The process occurs when an endogenous ribonuclease cleaves the longer dsRNA into shorter, e.g., 21- or 22-nucleotide-long RNAs, termed small interfering RNAs or siRNAs. The smaller RNA segments then mediate the degradation of the target mRNA. Kits for synthesis of RNAi are commercially available from, *e.g.* New England Biolabsor Ambion. In one embodiment one or more of the chemistries described above for use in antisense RNA can be employed in molecules that mediate RNAi.

As used herein, the term "immune response" includes immune cell-mediated (*e.g.*, T cell and/or B cell-mediated) immune responses that are influenced by modulation of immune cell activation. Exemplary immune responses include B cell responses (*e.g.*, antibody production, *e.g.*, IgA production), T cell responses (*e.g.*, proliferation, cytokine production and cellular cytotoxicity), and activation of cytokine responsive cells, *e.g.*, macrophages. In one embodiment of the invention, an immune response is T cell mediated. In another embodiment of the invention, an immune response is B cell mediated. As used herein, the term "downregulation" with reference to the immune response includes a diminution in any one or more immune responses, preferably T cell responses, while the term "upregulation" with reference to the immune response includes an increase in any one or more immune responses, preferably T cell responses. It will be understood that upregulation of one type of immune response may

lead to a corresponding downregulation in another type of immune response. For example, upregulation of the production of certain cytokines (*e.g.,* IL-10) can lead to downregulation of cellular immune responses. In addition, it will be understood that KRC may have one effect on immune responses in the context of T cell receptor-mediated signaling, another in the context of TNFα-mediated signaling, and another in the context of TGFβ-mediated signaling.

As used herein, the term "immune cell" includes cells that are of hematopoietic origin and that play a role in the immune response. immune cells include lymphocytes, such as B cells and T cells; natural killer cells; and myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

The terms "antigen presenting cell" and "APC", as used interchangeably herein, include professional antigen presenting cells (*e.g.,* B lymphocytes, monocytes, dendritic cells, and Langerhans cells) as well as other antigen presenting cells (*e.g.,* keratinocytes, endothelial cells, astrocytes, fibroblasts, and oligodendrocytes).

As used herein, the term "T cell" (*i.e.*, T lymphocyte) is intended to include all cells within the T cell lineage, including thymocytes, immature T cells, mature T cells and the like, from a mammal (*e.g.*, human). T cells include mature T cells that express either CD4 or CD8, but not both, and a T cell receptor. The various T cell populations described herein can be defined based on their cytokine profiles and their function.

As used herein "progenitor T cells" ("Thp") are pluripotent cells that express both CD4 and CD8.

As used herein, the term "naïve T cells" includes T cells that have not been exposed to cognate antigen and so are not activated or memory cells. Naïve T cells are not cycling and human naïve T cells are CD45RA+. If naïve T cells recognize antigen and receive additional signals depending upon but not limited to the amount of antigen, route of administration and timing of administration, they may proliferate and differentiate into various subsets of T cells, *e.g.,* effector T cells.

As used herein, the term "differentiated" refers to T cells that have been contacted with a stimulating agent and includes effector T cells (e.g., Th1, Th2) and memory T cells. Differentiated T cells differ in expression of several surface proteins compared to naïve T cells and secrete cytokines that activate other cells.

As used herein, the term "memory T cell" includes lymphocytes which, after exposure to antigen, become functionally quiescent and which are capable of surviving for long periods in the absence of antigen. Human memory T cells are CD45RA-.

As used herein, the term "effector T cell" includes T cells which function to eliminate antigen (*e.g.*, by producing cytokines which modulate the activation of other cells or by cytotoxic activity). The term "effector T cell" includes T helper cells (*e.g.*, Th1 and Th2 cells) and cytotoxic T cells. Th1 cells mediate delayed type hypersensitivity responses and macrophage activation while Th2 cells provide help to B cells and are critical in the allergic response (Mosmann and Coffman, 1989, *Annu. Rev. Immunol.* 7, 145-173; Paul and Seder, 1994, *Cell* 76, 241-251; Arthur and Mason, 1986, *J. Exp. Med.* 163, 774-786; Paliard *et al.*, 1988, *J. Immunol.* 141, 849-855; Finkelman *et al.*, 1988, *J. Immunol.* 141, 2335-2341). As used herein, the term " T helper type 1 response" (Th1 response) refers to a response that is characterized by the production of one or more cytokines selected from IFN-γ, IL-2, TNF, and lymphotoxin (LT) and other cytokines produced preferentially or exclusively by Th1 cells rather than by Th2 cells. As used herein, a "T helper type 2 response" (Th2 response) refers to a response by $CD4^+$ T cells that is characterized by the production of one or more cytokines selected from IL-4, IL-5, IL-6 and IL-10, and that is associated with efficient B cell "help" provided by the Th2 cells (*e.g.*, enhanced IgG1 and/or IgE production).

As used herein, the term "regulatory T cell" includes T cells which produce low levels of IL-2, IL-4, IL-5, and IL-12. Regulatory T cells produce TNFα, TGFβ, IFN-γ, and IL-10, albeit at lower levels than effector T cells. Although TGFβ is the predominant cytokine produced by regulatory T cells, the cytokine is produced at lower levels than in Th1 or Th2 cells, *e.g.*, an order of magnitude less than in Th1 or Th2 cells. Regulatory T cells can be found in the CD4+CD25+ population of cells (see, *e.g.*, Waldmann and Cobbold. 2001. *Immunity.* 14:399). Regulatory T cells actively suppress the proliferation and cytokine production of Th1, Th2, or naïve T cells which have been stimulated in culture with an activating signal (*e.g.*, antigen and antigen presenting cells or with a signal that mimics antigen in the context of MHC, *e.g.*, anti-CD3 antibody plus anti-CD28 antibody).

As used herein, the term "receptor" includes immune cell receptors that bind antigen, complexed antigen (*e.g.*, in the context of MHC molecules), or antibodies.

Activating receptors include T cell receptors (TCRs), B cell receptors (BCRs), cytokine receptors, LPS receptors, complement receptors, and Fc receptors. For example, T cell receptors are present on T cells and are associated with CD3 molecules. T cell receptors are stimulated by antigen in the context of MHC molecules (as well as by polyclonal T cell activating reagents). T cell activation via the TCR results in numerous changes, *e.g.,* protein phosphorylation, membrane lipid changes, ion fluxes, cyclic nucleotide alterations, RNA transcription changes, protein synthesis changes, and cell volume changes.

As used herein, the term "dominant negative" includes KRC molecules (*e.g.,* portions or variants thereof) that compete with native (*i.e.*, wild-type) KRC molecules, but which do not have KRC activity. Such molecules effectively decrease KRC activity in a cell.

As used herein, the term "inflammation" includes a response to injury which results in a dilation of the blood capillaries, a decrease in blood flow and an accumulation of leucocytes at the site of injury.

As used herein the term "apoptosis" includes programmed cell death which can be characterized using techniques which are known in the art. Apoptotic cell death can be characterized, *e.g.,* by cell shrinkage, membrane blebbing and chromatin condensation culminating in cell fragmentation. Cells undergoing apoptosis also display a characteristic pattern of internucleosomal DNA cleavage. As used herein, the term "modulating apoptosis" includes modulating programmed cell death in a cell, such as a epithelial cell. As used herein, the term "modulates apoptosis" includes either up regulation or down regulation of apoptosis in a cell. Modulation of apoptosis is discussed in more detail below and can be useful in ameliorating various disorders, *e.g.,* neurological disorders.

As used herein, the term "NFkB signaling pathway" refers to any one of the signaling pathways known in the art which involve activation or deactivation of the transcription factor NFkB, and which are at least partially mediated by the NFkB factor (Karin, 1998, *Cancer J from Scientific American*, 4:92-99; Wallach et al, 1999, *Ann Rev of Immunology*, 17:331-367). Generally, NFkB signaling pathways are responsive to a number of extracellular influences *e.g.* mitogens, cytokines, stress, and the like. The NFkB signaling pathways involve a range of cellular processes, including, but not

limited to, modulation of apoptosis. These signaling pathways often comprise, but are by no means limited to, mechanisms which involve the activation or deactivation via phosphorylation state of an inhibitor peptide of NFkB (IkB), thus indirectly activating or deactivating NFkB.

As used herein, the term "JNK signaling pathway" refers to any one of the signaling pathways known in the art which involve the Jun amino terminal kinase (JNK) (Karin, 1998, *Cancer J from Scientific American*, 4:92-99; Wallach et al, 1999, *Ann Rev of Immunology*, 17:331-367). This kinase is generally responsive to a number of extracellular signals *e.g.* mitogens, cytokines, stress, and the like. The JNK signaling pathways mediate a range of cellular processes, including, but not limited to, modulation of apoptosis. In a preferred embodiment, JNK activation occurs through the activity of one or more members of the TRAF protein family (See, *e.g.,* Wajant et al, 1999, *Cytokine Growth Factor Rev* 10:15-26).

As used herein, the term "TGFβ signaling pathway" refers to any one of the signaling pathways known in the art which involve transforming growth factor beta. A TGFβ signaling pathway is initiated when this molecule binds to and induces a heterodimeric cell-surface complex consisting of type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. This heterodimeric receptor then propagates the signal through phosphorylation of downstream target SMAD proteins. There are three functional classes of SMAD protein, receptor-regulated SMADs (R-SMADs), *e.g.,* SMAD2 and SMAD3, Co-mediator SMADs (Co-SMADs) and inhibitory SMADs (I-SMADs). Following phosphorylation by the heterodimeric receptor complex, the R-SMADs complex with the Co-SMAD and translocate to the nucleus, where in conjunction with other nuclear proteins, they regulate the transcription of target genes (Derynck, R., *et al.* (1998) *Cell* 95: 737-740). Reviewed in Massague, J. and Wotton, D. (2000) *EMBO J.* 19:1745.

The nucleotide sequence and amino acid sequnce of human SMAD2, is described in, for example, GenBank Accession No. gi:20127489. The nucleotide sequence and amino acid sequnce of murine SMAD2, is described in, for example, GenBank Accession No. gi:31560567. The nucleotide sequence and amino acid sequnce of human SMAD3, is described in, for example, GenBank Accession No.

gi:42476202. The nucleotide sequence and amino acid sequnce of murine SMAD3, is described in, for example, GenBank Accession No. gi:31543221.

"GATA3" is a Th2-specific transcription factor that is required for the development of Th2 cells. GATA-binding proteins constitute a family of transcription factors that recognize a target site conforming to the consensus WGATAR (W = A or T and R = A or G). GATA3 interacts with SMAD3 following its phosphorylation by TGFβ signaling to induce the differentiation of T helper cells. The nucleotide sequence and amino acid sequnce of human GATA3, is described in, for example, GenBank Accession Nos. gi:4503928 and gi:14249369. The nucleotide sequence and amino acid sequnce of murine GATA3, is described in, for example, GenBank Accession No. gi:40254638. The domains of GATA3 responsible for specific DNA-binding site recognition (amino acids 303 to 348) and trans activation (amino acids 30 to 74) have been identified. The signaling sequence for nuclear localization of human GATA-3 is a property conferred by sequences within and surrounding the amino finger (amino acids 249 to 311) of the protein. Exemplary genes whose transcription is regulated by GATA3 include IL-5, IL-12, IL-13, and IL-12Rβ2.

TGFß also plays a key role in osteoblast differentiation and bone development and remodeling. Osteoblasts secrete and deposit TGFß into the bone matrix and can respond to it, thus enabling possible autocrine modes of action. TGFß regulates the proliferation and differentiation of osteoblasts both in vitro and in vivo; however, the effects of TGFß on osteoblast differentiation depend on the extracellular milieu and the differentiation stage of the cells. TGFß stimulates proliferation and early osteoblast differentiation, while inhibiting terminal differentiation. Accordingly, TGFß has been reported to inhibit expression of alkaline phosphatase and osteocalcin, among other markers of osteoblast differentiation and function (Centrella et al., 1994 Endocr. Rev., 15, 27–39). Osteoblasts express cell surface receptors for TGFß and its known effectors, Smad2 and Smad3.

As used herein, "osteocalcin", also called bone Gla protein, is a vitamin K–dependent, calcium-binding bone protein, the most abundant noncollagen protein in bone. Osteocalcin is specifically expressed in differentiated osteoblasts and odontoblasts. The TGF-ß-mediated decrease of osteocalcin has been shown to occur at the mRNA level and does not require new protein synthesis. It has also been shown that

transcription from the osteocalcin promoter requires binding of the transcription factor CBFA1, also known as Runx2, to a response element, named OSE2, in the osteocalcin promoter.

Runx factors are DNA binding proteins that can facilitate tissue-specific gene activation or repression (Lutterbach, B., and S. W. Hiebert. (2000) Gene 245:223-235). Mammalian Runt-related genes are essential for blood, skeletal, and gastric development and are commonly mutated in acute leukemias and gastric cancers (Lund, A. H., and M. van Lohuizen. (2002) Cancer Cell. 1:213-215). Runx factors exhibit a tissue-restricted pattern of expression and are required for definitive hematopoiesis and osteoblast maturation. Runx proteins have recently been shown to interact through their C-terminal segment with Smads, a family of signaling proteins that regulate a diverse array of developmental and biological processes in response to transforming growth factor (TGF)-β/bone morphogenetic protein (BMP) family of growth factors. Moreover, subnuclear distribution of Runx proteins is mediated by the nuclear matrix-targeting signal, a protein motif present in the C terminus of Runx factors. Importantly, in vivo osteogenesis requires the C terminus of Runx2 containing the overlapping subnuclear targeting signal and the Smad interacting domain. The Runx and Smad proteins are jointly involved in the regulation of phenotypic gene expression and lineage commitment. Gene ablation studies have revealed that both Runx proteins and Smads are developmentally involved in hematopoiesis and osteogenesis. Furthermore, Runx2 and the BMP-responsive Smads can induce osteogenesis in mesenchymal pluripotent cells.

"Runx2" is one of three mammalian homologues of the Drosophila transcription factors Runt and Lozenge (Daga, A., et al.(1996) Genes Dev. 10:1194-1205). Runx2 is also expressed in T lymphocytes and cooperates with oncogenes c-myc, p53, and Pim1 to accelerate T-cell lymphoma development in mice (Blyth, K., et al. (2001) Oncogene 20:295-302).

Runx2 expression also plays a key role in osteoblast differentiation and skeletal formation. In addition to osteocalcin, Runx2 regulates expression of several other genes that are activated during osteoblast differentiation, including alkaline phosphatase, collagen, osteopontin, and osteoprotegerin ligand . These genes also contain Runx2 - binding sites in their promoters. These observations suggest that Runx2 is an essential

transcription factor for osteoblast differentiation. This hypothesis is strongly supported by the absence of bone formation in mouse embryos in which the cbfa1 gene was inactivated. Furthermore, cleidocranial dysplasia, a human disorder in which some bones are not fully developed, has been associated with mutations in a cbfa1 allele. In addition to its role in osteoblast differentiation, Runx2 has been implicated in the regulation of bone matrix deposition by differentiated osteoblasts. The expression of Runx2 is regulated by factors that influence osteoblast differentiation. Accordingly, BMPs can activate, while Smad2 and glucocorticoids can inhibit, Runx2 expression. In addition, Runx2 can bind to an OSE2 element in its own promoter, suggesting the existence of an autoregulatory feedback mechanism of transcriptional regulation during osteoblast differentiation. For a review, see, Alliston, et al.(2000) *EMBO J* 20:2254. The nucleotide sequence and amino acid sequnce of human Runx2, is described in, for example, GenBank Accession No. gi:10863884. The nucleotide sequence and amino acid sequnce of murine Runx2, is described in, for example, GenBank Accession No. gi:20806529.

As used herein, "AP-1" refers to the transcription factor activator protein 1 (AP-1) which is a family of DNA-binding factors that are composed of dimers of two proteins that bind to one another via a leucine zipper motif. The best characterized AP-1 factor comprises the proteins Fos and Jun. (Angel, P. and Karin, M. (1991) *Biochim. Biophys. Acta* 1072:129-157; Orengo, I. F. , Black, H. S. , *et al.* (1989) *Photochem. Photobiol.* 49:71-77; Curran, T. and Franza, B. R., Jr. (1988) *Cell* 55, 395-397). The AP-1 dimers bind to and transactivate promoter regions on DNA that contain cis-acting phorbol 12-tetradecanoate 13-acetate (TPA) response elements to induce transcription of genes involved in cell proliferation, metastasis, and cellular metabolism (Angel, P. , et al. (1987) Cell 49, 729-739. AP-1 is induced by a variety of stimuli and is implicated in the development of cancer and autoimmune disease. The nucleotide sequence and amino acid sequnce of human AP-1, is described in, for example, GenBank Accession No. gi:20127489.

As used herein, the term "nucleic acid" is intended to include fragments or equivalents thereof (e.g., fragments or equivalents thereof KRC, TRAF, c-Jun, c-Fos, GATA3, Runx2, SMAD, GLα). The term "equivalent" is intended to include nucleotide sequences encoding functionally equivalent KRC proteins, i.e., proteins which have the

ability to bind to the natural binding partner(s) of the KRC antigen. In a preferred embodiment, a functionally equivalent KRC protein has the ability to bind TRAF, *e.g.*, TRAF2, in the cytoplasm of an immune cell, *e.g.*, a T cell. In another preferred embodiment, a functionally equivalent KRC protein has the ability to bind Jun, *e.g.*, c-Jun, in the nucleoplasm of an immune cell, *e.g.*, a T cell. In another preferred embodiment, a functionally equivalent KRC protein has the ability to bind GATA3 in the nucleoplasm of an immune cell, *e.g.*, a T cell. In yet another preferred embodiment, a functionally equivalent KRC protein has the ability to bind SMAD, *e.g.*, SMAD2 and/or SMAD3, in the cytoplasm of an immune cell, *e.g.*, a B cell. In yet another preferred embodiment, a functionally equivalent KRC has the ability to bind Runx2 in the nucleoplasm of an immune cell, *e.g.*, a B cell.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid molecule is free of sequences which naturally flank the nucleic acid molecule (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid molecule) in the genomic DNA of the organism from which the nucleic acid molecule is derived.

As used herein, an "isolated protein" or "isolated polypeptide" refers to a protein or polypeptide that is substantially free of other proteins, polypeptides, cellular material and culture medium when isolated from cells or produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the KRC protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of KRC protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced.

The nucleic acids of the invention can be prepared, e.g., by standard recombinant DNA techniques. A nucleic acid of the invention can also be chemically synthesized

using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which has been automated in commercially available DNA synthesizers (See *e.g.,* Itakura *et al.* U.S. Patent No. 4,598,049; Caruthers *et al.* U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.,* bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.,* non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.,* replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid molecule of the invention, such as a recombinant expression vector of the invention, has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein. Preferably a host cell

is a mammalian cell, *e.g.*, a human cell. In particularly preferred embodiments, it is a epithelial cell.

As used herein, the term "transgenic cell" refers to a cell containing a transgene.

As used herein, a "transgenic animal" includes an animal, *e.g.*, a non-human mammal, *e.g.*, a swine, a monkey, a goat, or a rodent, *e.g.*, a mouse, in which one or more, and preferably essentially all, of the cells of the animal include a transgene. The transgene is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, *e.g.*, by microinjection, transfection or infection, *e.g.*, by infection with a recombinant virus. The term genetic manipulation includes the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA.

As used herein, the term "rodent" refers to all members of the phylogenetic order *Rodentia*.

As used herein, the term "misexpression" includes a non-wild type pattern of gene expression. Expression as used herein includes transcriptional, post transcriptional, *e.g.*, mRNA stability, translational, and post translational stages. Misexpression includes: expression at non-wild type levels, *i.e.*, over or under expression; a pattern of expression that differs from wild type in terms of the time or stage at which the gene is expressed, *e.g.*, increased or decreased expression (as compared with wild type) at a predetermined developmental period or stage; a pattern of expression that differs from wild type in terms of decreased expression (as compared with wild type) in a predetermined cell type or tissue type; a pattern of expression that differs from wild type in terms of the splicing size, amino acid sequence, post-translational modification, or biological activity of the expressed polypeptide; a pattern of expression that differs from wild type in terms of the effect of an environmental stimulus or extracellular stimulus on expression of the gene, *e.g.*, a pattern of increased or decreased expression (as compared with wild type) in the presence of an increase or decrease in the strength of the stimulus. Misexpression includes any expression from a transgenic nucleic acid. Misexpression includes the lack or non-expression of a gene or transgene, *e.g.*, that can be induced by a deletion of all or part of the gene or its control sequences.

As used herein, the term "knockout" refers to an animal or cell therefrom, in which the insertion of a transgene, *e.g.*, an exogenous DNA molecule, disrupts an

endogenous gene in the animal or cell therefrom. This disruption can essentially eliminate KRC in the animal or cell. In preferred embodiments, misexpression of the gene encoding the KRC protein is caused by disruption of the KRC gene. For example, the KRC gene can be disrupted through removal of DNA encoding all or part of the protein.

In preferred embodiments, the animal can be heterozygous or homozygous for a misexpressed KRC gene, *e.g.,* it can be a transgenic animal heterozygous or homozygous for a KRC transgene.

In preferred embodiments, the animal is a transgenic mouse with a transgenic disruption of the KRC gene, preferably an insertion or deletion, which inactivates the gene product.

In another aspect, the invention features, a nucleic acid molecule which, when introduced into an animal or cell, results in the misexpression of the KRC gene in the animal or cell. In preferred embodiments, the nucleic acid molecule, includes a KRC nucleotide sequence which includes a disruption, *e.g.,* an insertion or deletion and preferably the insertion of a marker sequence. The nucleotide sequence of the wild type KRC is known in the art and described in, for example, Mak, C.H., *et al.* (1998) *Immunogenetics* 48:32-39, the contents of which are incorporated herein by reference.

As used herein, the term "marker sequence" refers to a nucleic acid molecule that (a) is used as part of a nucleic acid construct (*e.g.,* the targeting construct) to disrupt the expression of the gene of interest (*e.g.,* the KRC gene) and (b) is used to identify those cells that have incorporated the targeting construct into their genome. For example, the marker sequence can be a sequence encoding a protein which confers a detectable trait on the cell, such as an antibiotic resistance gene, *e.g.,* neomycin resistance gene, or an assayable enzyme not typically found in the cell, *e.g.,* alkaline phosphatase, horseradish peroxidase, luciferase, beta-galactosidase and the like.

As used herein, "disruption of a gene" refers to a change in the gene sequence, *e.g.,* a change in the coding region. Disruption includes: insertions, deletions, point mutations, and rearrangements, *e.g.,* inversions. The disruption can occur in a region of the native KRC DNA sequence (*e.g.,* one or more exons) and/or the promoter region of the gene so as to decrease or prevent expression of the gene in a cell as compared to the wild-type or naturally occurring sequence of the gene. The "disruption" can be induced

by classical random mutation or by site directed methods. Disruptions can be transgenically introduced. The deletion of an entire gene is a disruption. Preferred disruptions reduce KRC levels to about 50% of wild type, in heterozygotes or essentially eliminate KRC in homozygotes.

As used herein, the term "antibody" is intended to include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which binds (immunoreacts with) an antigen, such as Fab and $F(ab')_2$ fragments, single chain antibodies, intracellular antibodies, scFv, Fd, or other fragments. Preferably, antibodies of the invention bind specifically or substantially specifically to KRC, TRAF, c-Jun, c-Fos, GATA3, SMAD, or Runx2, molecules (*i.e.*, have little to no cross reactivity with non-KRC, non-TRAF, non-c-Jun, non-c-Fos, non-GATA3, non-SMAD, or non-Runx2, molecules). The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal antibodies" and "polyclonal antibody composition" refer to a population of antibody molecules that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody compositions thus typically display a single binding affinity for a particular antigen with which it immunoreacts.

As used herein, the term "disorders that would benefit from the modulation of KRC activity or expression" or "KRC associated disorder" includes disorders in which KRC activity is aberrant or which would benefit from modulation of a KRC activity. Preferably, KRC associated disorders involve aberrant proliferation of cells, *e.g.*, excessive or unwanted proliferation of cells or deficient proliferation of cells. In one embodiment, KRC associated disorders are disorders such ás inflammation. Examples of KRC associated disorders include: disorders involving aberrant or unwanted proliferation of cells, *e.g.*, inflammation, autoimmunity, neoplasia, or cell death, e.g., apoptosis, or necrosis. KRC associated disorders may also include disorders that have been linked generally to aberrant TGFβ activity or function, including, for example, B cell chronic lymphocytic leukemia (B-CLL). Further examples of KRC associated disorders include carcinomas, adenocarcinomas, leukemias, lymphomas, and other

neoplasias. KRC disorders may also include disorders that have been linked generally to aberrant TNF receptor activity or function, including Crohn's Disease (Baert and Rutgeerts, 1999, *Int J Colorectal Dis*, 14:47-51) and certain cardiovascular diseases (Ferrari, 1999, *Pharmacol Res*, 40:97-105). They may also include disorders characterized by uncontrolled or aberrant levels of apoptosis, for example myelokathexis (Aprikyan *et al.*, 2000, *Blood*, 95:320-327), and autoimmune lymphoproliferative syndrome (Jackson and Puck, 1999, *Curr Op Pediatr*, 11:521-527; Straus *et al.*, 1999, *Ann Intern Med*, 130:591-601). KRC associated disorders may also include metabolic bone disorders, such as, but not limited to, osteoporosis, osteomalacia, skeletal changes of hyperparathyroidism and chronic renal failure (renal osteodystrophy) and osteitis deformans (Paget's disease of bone).

In one embodiment, small molecules can be used as test compounds. The term "small molecule" is a term of the art and includes molecules that are less than about 7500, less than about 5000, less than about 1000 molecular weight or less than about 500 molecular weight. In one embodiment, small molecules do not exclusively comprise peptide bonds. In another embodiment, small molecules are not oligomeric. Exemplary small molecule compounds which can be screened for activity include, but are not limited to, peptides, peptidomimetics, nucleic acids, carbohydrates, small organic molecules (e.g., Cane et al. 1998. Science 282:63), and natural product extract libraries.. In another embodiment, the compounds are small, organic non-peptidic compounds. In a further embodiment, a small molecule is not biosynthetic. For example, a small molecule is preferably not itself the product of transcription or translation.

Various aspects of the invention are described in further detail below:

II. Screening Assays to Identify KRC Modulating Agents

Modulators of KRC activity can be known (*e.g.*, dominant negative inhibitors of KRC activity, antisense KRC intracellular antibodies that interfere with KRC activity, peptide inhibitors derived from KRC) or can be identified using the methods described herein. The invention provides methods (also referred to herein as "screening assays") for identifying other modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptidomimetics, small molecules or other drugs) which modulate KRC activity and for testing or optimizing the activity of other agents.

For example, in one embodiment, molecules which bind, e.g., to KRC or a molecule in a signaling pathway involving KRC (e.g., TRAF, NF-kB, JNK, GATA3, SMAD, Runx2, or AP-1)or have a stimulatory or inhibitory effect on the expression and or activity of KRC or a molecule in a signal transduction pathway involving KRC can be identified. For example, c-Jun, NF-kB, TRAF, GATA3, SMAD, Runx2, and JNK function in a signal transduction pathway involving KRC, therefore, any of these molecules can be used in the subject screening assays. Although the specific embodiments described below in this section and in other sections may list one of these molecules as an example, other molecules in a signal transduction pathway involving KRC can also be used in the subject screening assays.

In one embodiment, the ability of a compound to directly modulate the expression, post-translational modification (*e.g.*, phosphorylation), or activity of KRC is measured in an indicator composition using a screening assay of the invention.

The indicator composition can be a cell that expresses the KRC protein or a molecule in a signal transduction pathway involving KRC, for example, a cell that naturally expresses or, more preferably, a cell that has been engineered to express the protein by introducing into the cell an expression vector encoding the protein. Preferably, the cell is a mammalian cell, e.g., a human cell. In one embodiment, the cell is a T cell. In another embodiment, the cell is a B cell. In another embodiment, the cell is a osteoblast. Alternatively, the indicator composition can be a cell-free composition that includes the protein (*e.g.*, a cell extract or a composition that includes e.g., either purified natural or recombinant protein).

Compounds identified using the assays described herein can be useful for treating disorders associated with aberrant expression, post-translational modification, or activity of KRC or a molecule in a signaling pathway involving KRC *e.g*: disorders that would benefit from modulation of TNFα production, modulation of IL-2 production, modulation of a JNK signaling pathway, modulation of an NFkB signaling pathway, modulation of a TGFβ signaling pathway, modulation of AP-1 activity, modulation of Ras and Rac activity, modulation of actin polymerization, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF, modulation of ubiquitination of Runx2, modulation of the degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD, modulation of degradation of Runx2, modulation

of degradation of GATA3, modulation of GATA3 expression, modulation of Th2 cell differentiation, modulation of Th2 cytokine production, modulation of IgA production, modulation of GLα transcription (Igα chain germline transcription), and/or modulation of osteocalcin gene transcription.

Conditions that can benefit from modulation of a signal transduction pathway involving KRC include autoimmune disorders as well as malignancies, immunodeficiency disorders and metabolic bone diseases.. Compounds which modulate KRC expression and/or activity can also be used to modulate the immune response.

The subject screening assays can be performed in the presence or absence of other agents. In one embodiment, the subject assays are performed in the presence of an agent that provides a T cell receptor-mediated signal. In another embodiment, the subject assays are performed in the presence of an agent that provides a B cell receptor-mediated signal

In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulating agent can be identified using a cell-based or a cell-free assay, and the ability of the agent to modulate the activity of KRC or a molecule in a signal transduction pathway involving KRC can be confirmed *in vivo*, *e.g.*, in an animal such as an animal model for multiple myeloma, neoplastic diseases, renal cell carcinoma, B-CLL, metabolic bone disease, or autoimmune diseases.

Moreover, a modulator of KRC or a molecule in a signaling pathway involving KRC identified as described herein (*e.g.*, an antisense nucleic acid molecule, or a specific antibody, or a small molecule) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such a modulator. Alternatively, a modulator identified as described herein can be used in an animal model to determine the mechanism of action of such a modulator.

In another embodiment, it will be understood that similar screening assays can be used to identify compounds that indirectly modulate the activity and/or expression of KRC *e.g.*, by performing screening assays such as those described above using molecules with which KRC interacts, *e.g.*, molecules that act either upstream or downstream of KRC in a signal transduction pathway.

The cell based and cell free assays of the invention are described in more detail below.

A. Cell Based Assays

The indicator compositions of the invention can be cells that expresses at least one of a KRC protein or non-KRC protein in the KRC signaling pathway (such as, e.g., TRAF, NF-kB, JNK, Jun, TGFβ, GATA3, SMAD, Runx2, or AP-1) for example, a cell that naturally expresses the endogenous molecule or, more preferably, a cell that has been engineered to express an exogenous KRC, TRAF, NF-kB, JNK, Jun, TGFβ, GATA3, SMAD, Runx2, or AP-1 protein by introducing into the cell an expression vector encoding the protein(s). Alternatively, the indicator composition can be a cell-free composition that includes at least one of a KRC or a non- KRC protein such as TRAF, NF-kB, JNK, Jun, TGFβ, GATA3, SMAD, Runx2, or AP-1 (*e.g.*, a cell extract from a cell expressing the protein or a composition that includes purified KRC, TRAF, NF-kB, JNK, Jun, TGFβ, GATA3, SMAD, Runx2, or AP-1 protein, either natural or recombinant protein).

Compounds that modulate expression and/or activity of KRC, or a non-KRC protein that acts upstream or downstream of can be identified using various "read-outs."

For example, an indicator cell can be transfected with an expression vector, incubated in the presence and in the absence of a test compound, and the effect of the compound on the expression of the molecule or on a biological response regulated by can be determined. The biological activities of include activities determined *in vivo*, or *in vitro*, according to standard techniques. Activity can be a direct activity, such as an association with a target molecule or binding partner (*e.g.*, a protein such as the Jun, *e.g.*, c-Jun, TRAF, *e.g.*, TRAF2, GATA3, SMAD, *e.g.*, SMAD2, SMAD3, protein. Alternatively, activity is an indirect activity, such as a cellular signaling activity occurring downstream of the interaction of the protein with an target molecule or a biological effect occurring as a result of the signaling cascade triggered by that interaction. For example, biological activities of KRC described herein include: modulation of TNFα production, modulation of IL-2 production, modulation of a JNK signaling pathway, modulation of an NFkB signaling pathway, modulation of a TGFβ signaling pathway, modulation of AP-1 activity, modulation of Ras and Rac activity, modulation of actin polymerization, modulation of ubiquitination of AP-1, modulation of ubiquitination of TRAF2, modulation of ubiquitination of Runx2, modulation of the

degradation of c-Jun, modulation of the degradation of c-Fos, modulation of degradation of SMAD3, modulation of degradation of Runx 2, modulation of degradation of GATA3, modulation of effector T cell function, modulation of T cell anergy, modulation of apoptosis, or modulation of T cell differentiation, and/or modulation of IgA germline transcription.

To determine whether a test compound modulates KRC protein expression, *in vitro* transcriptional assays can be performed. In one example of such an assay, a regulatory sequence (eg., the full length promoter and enhancer) of KRC can be operably linked to a reporter gene such as chloramphenicol acetyltransferase (CAT), GFP, or luciferase and introduced into host cells. Other techniques are known in the art.

As used interchangeably herein, the terms "operably linked" and "operatively linked" are intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence in a host cell (or by a cell extract). Regulatory sequences are art-recognized and can be selected to direct expression of the desired protein in an appropriate host cell. The term regulatory sequence is intended to include promoters, enhancers, polyadenylation signals and other expression control elements. Such regulatory sequences are known to those skilled in the art and are described in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transfected and/or the type and/or amount of protein desired to be expressed.

A variety of reporter genes are known in the art and are suitable for use in the screening assays of the invention. Examples of suitable reporter genes include those which encode chloramphenicol acetyltransferase, beta-galactosidase, alkaline phosphatase, green fluorescent protein, or luciferase. Standard methods for measuring the activity of these gene products are known in the art.

A variety of cell types are suitable for use as an indicator cell in the screening assay. Preferably a cell line is used which expresses low levels of endogenous KRC (or, e.g., TRAF, Fos, Jun, NF-kB, TGFβ, GATA3, SMAD, and/or Runx2) and is then engineered to express recombinant protein. Cells for use in the subject assays include both eukaryotic and prokaryotic cells. For example, in one embodiment, a cell is a bacterial cell. In another embodiment, a cell is a fungal cell, such as a yeast cell. In

another embodiment, a cell is a vertebrate cell, *e.g.*, an avian cell or a mammalian cell (*e.g.*, a murine cell, or a human cell).

In one embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is higher than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that stimulates the expression of KRC (or, e.g., TRAF, Fos, Jun, NF-kB, TGFβ, GATA3, SMAD, and/or Runx2). In another embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is lower than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that inhibits the expression of KRC (or, e.g., TRAF, Fos, Jun, NF-kB, TGFβ, GATA3, SMAD, and/or Runx2).

In one embodiment, the invention provides methods for identifying compounds that modulate cellular responses in which KRC is involved.

In one embodiment differentiation of cells, e.g., T cells, can be used as an indicator of modulation of KRC or a signal transduction pathway involving KRC. Cell differentiation can be monitored directly (*e.g.* by microscopic examination of the cells for monitoring cell differentiation), or indirectly, *e.g.*, by monitoring one or more markers of cell differentiation (*e.g.,* an increase in mRNA for a gene product associated with cell differentiation, or the secretion of a gene product associated with cell differentiation, such as the secretion of a protein (*e.g.*, the secretion of cytokines) or the expression of a cell surface marker (such as CD69). Standard methods for detecting mRNA of interest, such as reverse transcription-polymerase chain reaction (RT-PCR) and Northern blotting, are known in the art. Standard methods for detecting protein secretion in culture supernatants, such as enzyme linked immunosorbent assays (ELISA), are also known in the art. Proteins can also be detected using antibodies, *e.g.*, in an immunoprecipitation reaction or for staining and FACS analysis.

In another embodiment, the ability of a compound to modulate effector T cell function can be determined. For example, in one embodiment, the ability of a compound to modulate T cell proliferation, cytokine production, and/or cytotoxicity can be measured using techniques well known in the art.

In one embodiment, the ability of a compound to modulate IL-2 production can be determined. Production of IL-2 can be monitored, for example, using Northern or Western blotting. IL-2 can also be detected using an ELISA assay or in a bioassay, *e.g.*, employing cells which are responsive to IL-2 (*e.g.*, cells which proliferate in response to the cytokine or which survive in the presence of the cytokine) using standard techniques.

In another embodiment, the ability of a compound to modulate apoptosis can be determined. Apoptosis can be measured in the presence or the absence of Fas-mediated signals. In one embodiment, cytochrome C release from mitochondria during cell apoptosis can be detected, *e.g.*, plasma cell apoptosis (as described in, for example, Bossy-Wetzel E. *et al.* (2000) *Methods in Enzymol.* 322:235-42). Other exemplary assays include: cytofluorometric quantitation of nuclear apoptosis induced in a cell-free system (as described in, for example, Lorenzo H.K. *et al.* (2000) *Methods in Enzymol.* 322:198-201); apoptotic nuclease assays (as described in, for example, Hughes F.M. (2000) *Methods in Enzymol.* 322:47-62); analysis of apoptotic cells, *e.g.*, apoptotic plasma cells, by flow and laser scanning cytometry (as described in, for example, Darzynkiewicz Z. *et al.* (2000) *Methods in Enzymol.* 322:18-39); detection of apoptosis by annexin V labeling (as described in, for example, Bossy-Wetzel E. *et al.* (2000) *Methods in Enzymol.* 322:15-18); transient transfection assays for cell death genes (as described in, for example, Miura M. *et al.* (2000) *Methods in Enzymol.* 322:480-92); and assays that detect DNA cleavage in apoptotic cells, *e.g.*, apoptotic plasma cells (as described in, for example, Kauffman S.H. *et al.* (2000) *Methods in Enzymol.* 322:3-15). Apoptosis can also be measured by propidium iodide staining or by TUNEL assay. In another embodiment, the transcription of genes associated with a cell signaling pathway involved in apoptosis (*e.g.*, JNK) can be detected using standard methods.

In another embodiment, mitochondrial inner membrane permeabilization can be measured in intact cells by loading the cytosol or the mitochondrial matrix with a die that does not normally cross the inner membrane, e.g., calcein (Bernardi et al. 1999. Eur. J. Biochem. 264:687; Lemasters, J., J. et al. 1998. Biochem. Biophys. Acta 1366:177. In another embodiment, mitochondrial inner membrane permeabilization can be assessed, e.g., by determining a change in the mitochondrial inner membrane potential ($\Delta\Psi m$). For example, cells can be incubated with lipophilic cationic fluorochromes such as DiOC6 (Gross et al. 1999. Genes Dev. 13:1988)

(3,3'dihexyloxacarbocyanine iodide) or JC-1 (5,5',6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide). These dyes accumulate in the mitochondrial matrix, driven by the Ψm . Dissipation results in a reduction of the fluorescence intensity (e.g., for DiOC6 (Gross et al. 1999. Genes Dev. 13:1988) or a shift in the emission spectrum of the dye. These changes can be measured by cytofluorometry or microscopy.

In yet another embodiment, the ability of a compound to modulate translocation of KRC to the nucleus can be determined. Translocation of KRC to the nucleus can be measured, e.g., by nuclear translocation assays in which the emission of two or more fluorescently-labeled species is detected simultaneously. For example, the cell nucleus can be labeled with a known fluorophore specific for DNA, such as Hoechst 33342. The KRC protein can be labeled by a variety of methods, including expression as a fusion with GFP or contacting the sample with a fluorescently-labeled antibody specific for KRC. The amount KRC that translocates to the nucleus can be determined by determining the amount of a first fluorescently-labeled species, i.e., the nucleus, that is distributed in a correlated or anti-correlated manner with respect to a second fluorescently-labeled species, i.e., KRC, as described in U.S. Patent No. 6,400,487, the contents of which are hereby incorporated by reference.

In one embodiment, the effect of a compound on a JNK signaling pathway can be determined. The JNK group of MAP kinases is activated by exposure of cells to environmental stress or by treatment of cells with pro-inflammatory cytokines. A combination of studies involving gene knockouts and the use of dominant-negative mutants have implicated both MKK4 and MKK7 in the phosphorylation and activation of JNK. Targets of the JNK signal transduction pathway include the transcription factors ATF2 and c-Jun. JNK binds to an NH_2-terminal region of ATF2 and c-Jun and phosphorylates two sites within the activation domain of each transcription factor, leading to increased transcriptional activity. JNK is activated by dual phosphorylation on Thr-183 and Tyr-185. To determine the effect of a compound on a JNK signal transduction pathway, the ability of the compound to modulate the activation status of various molecules in the signal transduction pathway can be determined using standard techniques. For example, in one embodiment, the phosphorylation status of JNK can be

examined by immunoblotting with the anti-ACTIVE-JNK antibody (Promega), which specifically recognizes the dual phosphorylated TPY motif.

In another embodiment, the effect of a compound on an NFkB signal transduction pathway can be determined. The ability of the compound to modulate the activation status of various components of the NFkB pathway can be determined using standard techniques. NFkB constitutes a family of Rel domain-containing transcription factors that play essential roles in the regulation of inflammatory, anti-apoptotic, and immune responses. The function of the NFkB/Rel family members is regulated by a class of cytoplasmic inhibitory proteins termed IBs that mask the nuclear localization domain of NFkB causing its retention in the cytoplasm. Activation of NFkB by TNF-α and IL-1 involves a series of signaling intermediates, which may converge on the NFkB-inducing kinase (NIK). This kinase in turn activates the IB kinase (IKK) isoforms. These IKKs phosphorylate the two regulatory serines located in the N termini of IB molecules, triggering rapid ubiquitination and degradation of IB in the 26S proteasome complex. The degradation of IB unmasks a nuclear localization signal present in the NFkB complex, allowing its rapid translocation into the nucleus, where it engages cognate B enhancer elements and modulates the transcription of various NFkB-responsive target genes. In one embodiment, the ability of a compound to modulate one or more of: the status of NFkB inhibitors, the ability of NFkB to translocate to the nucleus, or the activation of NFkB dependent gene transcription can be measured.

In one embodiment, the ability of a compound to modulate AP-1 activity can be measured. The AP-1 complex is comprised of the transcription factors Fos and Jun. The AP-1 complex activity is controlled by regulation of Jun and Fos transcription and by posttranslation modification, for example, the activation of several MAPKS, ERK, p38 and JN, is required for AP-1 transcriptional activity. In one embodiment, the modulation of transcription mediated by AP-1 can be measured. In another embodiment, the ability of a compound to modulate the activity of AP-1, e.g., by modulating its phosphorylation or its ubiquitination can be measured. In one embodiment, the ubiquitination of AP-1 can be measured using techniques known in the art. In another embodiment, the degradation of AP-1 (or of c-Jun and/or c-Fos) can be measured using known techniques.

The loss of AP-1 has been associated with T cell anergy. Accordingly, in one embodiment, the ability of a test compound to modulate T cell anergy can be determined, e.g, by assaying secondary T cell responses. If the T cells are unresponsive to the secondary activation attempts, as determined by IL-2 synthesis and/or T cell proliferation, a state of anergy or has been induced. Standard assay procedures can be used to measure T cell anergy, for example, T cell proliferation can be measured, for example, by assaying [^{3}H] thymidine incorporation. In another embodiment, signal transduction can be measured, e.g., activation of members of the MAP kinase cascade or activation of the AP-1 complex can be measured. In another embodiment, intracellular calcium mobilization, protein levels members of the NFAT cascade can be measured.

In another embodiment, the effect of a compound on Ras and Rac activity can be measured using standard techniques. In one embodiment, actin polymerization, e.g., by measuring the immunofluorescence of F-actin can be measured.

In another embodiment, the effect of the compound on ubiquitination of, for example, AP1, SMAD, TRAF, and/or Runx2, can be measured, by, for example in vitro or in vivo ubiquitination assays. In vitro ubiquitination assays are described in, for example, Fuchs, S. Y., Bet al. (1997) J. Biol. Chem. 272:32163-32168. In vivo ubiquitination assays are described in, for example, Treier, M., L. et al. (1994) Cell 78:787-798.

In another embodiment, the effect of the compound on the degradation of, for example, a KRC target molecule and/or a KRC binding partner, can be measured by, for example, coimmunoprecipitation of KRC, *e.g.*, full-length KRC and/or a fragment thereof, with, *e.g.*, SMAD, GATA3, Runx2, TRAF, Jun, and/or Fos. Western blotting of the coimmunoprecipitate and probing of the blots with antibodies to KRC and the KRC target molecule and/or a KRC binding partner can be quantitated to determine whether degradation has occurred.

In one embodiment, the ability of the compound to modulate TGFβ signaling in B cells can be measured. For example, as described herein, KRC is a coactivator of GLα promoter activity and a corepressor of the osteocalcin gene. In the absence of KRC, GLα transcription is diminished in B cells, and osteocalcin gene transcription is augmented in osteoblasts. Accordingly, in one embodiment, the ability of the compound to modulate TGFβ signaling in B cells can be measured by measuring the transcription of GLα. In

another embodiment, osteocalcin gene transcription can be measured. In one embodiment, RT-PCR is used to measure the transcritpion. Furthermore, given the ability of KRC to interact with SMAD and drive the transcription of a SMAD reporter construct, the ability of a compound to modulate TGFβ signaling in B cells can be measured by measuring the transcriptional ability of SMAD. In one embodiment, SMAD, or a fragment thereof, *e.g.,* a basic SMAD-binding element, is operably linked to a luciferase reporter gene. Other TGFβ regulated genes are known in the art (e.g., Massague and Wotton. 2000 EMBO 19:1745.)

The ability of the test compound to modulate KRC (or a molecule in a signal transduction pathway involving to KRC) binding to a substrate or target molecule (e.g., TRAF, GATA3, SMAD, Runx2, or Jun in the case of KRC) can also be determined. Determining the ability of the test compound to modulate KRC binding to a target molecule (*e.g.*, a binding partner such as a substrate) can be accomplished, for example, by coupling the target molecule with a radioisotope or enzymatic label such that binding of the target molecule to KRC or a molecule in a signal transduction pathway involving KRC can be determined by detecting the labeled KRC target molecule in a complex. Alternatively, KRC be coupled with a radioisotope or enzymatic label to monitor the ability of a test compound to modulate KRC binding to a target molecule in a complex. Determining the ability of the test compound to bind to KRC can be accomplished, for example, by coupling the compound with a radioisotope or enzymatic label such that binding of the compound to KRC can be determined by detecting the labeled compound in a complex. For example, targets can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, compounds can be labeled, e.g., with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

In another embodiment, the ability of KRC or a molecule in a signal transduction pathway involving KRC to be acted on by an enzyme or to act on a substrate can be measured. For example, in one embodiment, the effect of a compound on the phosphorylation of KRC can be measured using techniques that are known in the art.

It is also within the scope of this invention to determine the ability of a compound to interact with KRC or a molecule in a signal transduction pathway involving KRC without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a compound with a KRC molecule without the labeling of either the compound or the molecule (McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912). As used herein, a "microphysiometer" (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a compound and

Exemplary target molecules of KRC include: Jun, TRAF (e.g., TRAF2) GATA3, SMAD, *e.g.,* SMAD2 and SMAD3, and Runx2.

In another embodiment, a different (*i.e.*, non-KRC) molecule acting in a pathway involving KRC that acts upstream or downstream of KRC can be included in an indicator composition for use in a screening assay. Compounds identified in a screening assay employing such a molecule would also be useful in modulating KRC activity, albeit indirectly. For example, the ability of TRAF (e.g., TRAF2) to activate NFKβ dependent gene expression can be measured, or the ability of SMAD to activate TGFβ-dependent gene transcription can be measured..

The cells used in the instant assays can be eukaryotic or prokaryotic in origin. For example, in one embodiment, the cell is a bacterial cell. In another embodiment, the cell is a fungal cell, *e.g.*, a yeast cell. In another embodiment, the cell is a vertebrate cell, *e.g.*, an avian or a mammalian cell. In a preferred embodiment, the cell is a human cell.

The cells of the invention can express endogenous KRC or another protein in a signaling pathway involving KRC or can be engineered to do so. For example, a cell that has been engineered to express the KRC protein and/or a non protein which acts upstream or downstream of can be produced by introducing into the cell an expression vector encoding the protein.

Recombinant expression vectors that can be used for expression of KRC or a molecule in a signal transduction pathway involving KRC (e.g., a protein which acts upstream or downstream of KRC) are known in the art. For example, the cDNA is first introduced into a recombinant expression vector using standard molecular biology

techniques. A cDNA can be obtained, for example, by amplification using the polymerase chain reaction (PCR) or by screening an appropriate cDNA library. The nucleotide sequences of cDNAs for or a molecule in a signal transduction pathway involving (*e.g.*, human, murine and yeast) are known in the art and can be used for the design of PCR primers that allow for amplification of a cDNA by standard PCR methods or for the design of a hybridization probe that can be used to screen a cDNA library using standard hybridization methods.

Following isolation or amplification of a cDNA molecule encoding KRC or a non-KRC molecule in a signal transduction pathway involving KRC the DNA fragment is introduced into an expression vector. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid molecule in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression and the level of expression desired, which is operatively linked to the nucleic acid sequence to be

expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell, those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences) or those which direct expression of the nucleotide sequence only under certain conditions (*e.g.*, inducible regulatory sequences).

When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma virus, adenovirus, cytomegalovirus and Simian Virus 40. Non-limiting examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987), *EMBO J. 6*:187-195). A variety of mammalian expression vectors carrying different regulatory sequences are commercially available. For constitutive expression of the nucleic acid in a mammalian host cell, a preferred regulatory element is the cytomegalovirus promoter/enhancer. Moreover, inducible regulatory systems for use in mammalian cells are known in the art, for example systems in which gene expression is regulated by heavy metal ions (see *e.g.*, Mayo *et al.* (1982) *Cell* 29:99-108; Brinster *et al.* (1982) *Nature* 296:39-42; Searle *et al.* (1985) *Mol. Cell. Biol.* 5:1480-1489), heat shock (see *e.g.*, Nouer *et al.* (1991) in *Heat Shock Response*, e.d. Nouer, L. , CRC, Boca Raton , FL, pp167-220), hormones (see *e.g.*, Lee *et al.* (1981) *Nature* 294:228-232; Hynes *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2038-2042; Klock *et al.* (1987) *Nature* 329:734-736; Israel & Kaufman (1989) *Nucl. Acids Res.* 17:2589-2604; and PCT Publication No. WO 93/23431), FK506-related molecules (see *e.g.*, PCT Publication No. WO 94/18317) or tetracyclines (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT

Publication No. WO 96/01313). Still further, many tissue-specific regulatory sequences are known in the art, including the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916) and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

Vector DNA can be introduced into mammalian cells via conventional transfection techniques. As used herein, the various forms of the term "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into mammalian host cells, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on a separate vector from that encoding KRC or, more preferably, on the same vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

In one embodiment, within the expression vector coding sequences are operatively linked to regulatory sequences that allow for constitutive expression of the molecule in the indicator cell (*e.g.*, viral regulatory sequences, such as a cytomegalovirus promoter/enhancer, can be used). Use of a recombinant expression vector that allows for constitutive expression of KRC or a molecule in a signal transduction pathway involving KRC in the indicator cell is preferred for identification of compounds that enhance or inhibit the activity of the molecule. In an alternative embodiment, within the expression vector the coding sequences are operatively linked to regulatory sequences of the endogenous gene for KRC or a molecule in a signal transduction pathway involving KRC (*i.e.*, the promoter regulatory region derived from the endogenous gene). Use of a recombinant expression vector in which expression is controlled by the endogenous regulatory sequences is preferred for identification of compounds that enhance or inhibit the transcriptional expression of the molecule.

In yet another aspect of the invention, the KRC protein or fragments thereof can be used as "bait protein" e.g., in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with KRC ("binding proteins" or " bp") and are involved in KRC activity. Such KRC -binding proteins are also likely to be involved in the propagation of signals by the KRC proteins or KRC targets such as, for example, downstream elements of an KRC-mediated signaling pathway. Alternatively, such KRC -binding proteins can be KRC inhibitors.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for an KRC protein is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo,* forming an KRC dependent complex, the DNA-binding and activation domains of the transcription factor

are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the KRC protein or a molecule in a signal transduction pathway involving KRC.

B. Cell-free assays

In another embodiment, the indicator composition is a cell free composition. KRC or a non- KRC protein in a signal transduction pathway involving KRC expressed by recombinant methods in a host cells or culture medium can be isolated from the host cells, or cell culture medium using standard methods for protein purification. For example, ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies can be used to produce a purified or semi-purified protein that can be used in a cell free composition. Alternatively, a lysate or an extract of cells expressing the protein of interest can be prepared for use as cell-free composition.

In one embodiment, compounds that specifically modulate KRC activity or the activity of a molecule in a signal transduction pathway involving KRC are identified based on their ability to modulate the interaction of KRC with a target molecule to which KRC binds. The target molecule can be a DNA molecule, *e.g.*, an KRC - responsive element, such as the regulatory region of a chaperone gene) or a protein molecule. Suitable assays are known in the art that allow for the detection of protein-protein interactions (*e.g.*, immunoprecipitations, two-hybrid assays and the like) or that allow for the detection of interactions between a DNA binding protein with a target DNA sequence (*e.g.*, electrophoretic mobility shift assays, DNAse I footprinting assays and the like). By performing such assays in the presence and absence of test compounds, these assays can be used to identify compounds that modulate (*e.g.*, inhibit or enhance) the interaction of KRC with a target molecule.

In one embodiment, the amount of binding of KRC or a molecule in a signal transduction pathway involving KRC to the target molecule in the presence of the test compound is greater than the amount of binding of KRC to the target molecule in the absence of the test compound, in which case the test compound is identified as a

compound that enhances binding of KRC to a target. In another embodiment, the amount of binding of the KRC to the target molecule in the presence of the test compound is less than the amount of binding of the KRC (or e.g., Jun, TRAF, GATA3, SMAD, Runx2) to the target molecule in the absence of the test compound, in which case the test compound is identified as a compound that inhibits binding of KRC to the target. Binding of the test compound to KRC or a molecule in a signal transduction pathway involving KRC can be determined either directly or indirectly as described above. Determining the ability of KRC protein to bind to a test compound can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA) (Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345; Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705). As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In the methods of the invention for identifying test compounds that modulate an interaction between KRC (or e.g., Jun, TRAF, GATA3, SMAD, Runx2) protein and a target molecule, the complete KRC protein can be used in the method, or, alternatively, only portions of the protein can be used. For example, an isolated KRC interacting domain (e.g., consisting of amino acids 204-1055 or a larger subregion including an interacting domain) can be used. An assay can be used to identify test compounds that either stimulate or inhibit the interaction between the KRC protein and a target molecule. A test compound that stimulates the interaction between the protein and a target molecule is identified based upon its ability to increase the degree of interaction between, e.g., KRC and a target molecule as compared to the degree of interaction in the absence of the test compound and such a compound would be expected to increase the activity of KRC in the cell. A test compound that inhibits the interaction between the protein and a target molecule is identified based upon its ability to decrease the degree of interaction between the protein and a target molecule as compared to the degree of interaction in the absence of the compound and such a compound would be expected to decrease KRC activity.

In one embodiment of the above assay methods of the present invention, it may be desirable to immobilize either KRC (or a molecule in a signal transduction pathway

involving KRC, e.g., Jun, TRAF, GATA3, SMAD, Runx2) or a respective target molecule for example, to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, or to accommodate automation of the assay. Binding of a test compound to a KRC or a molecule in a signal transduction pathway involving KRC, or interaction of an KRC protein (or a molecule in a signal transduction pathway involving KRC) with a target molecule in the presence and absence of a test compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided in which a domain that allows one or both of the proteins to be bound to a matrix is added to one or more of the molecules. For example, glutathione-S-transferase fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or KRC protein, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix is immobilized in the case of beads, and complex formation is determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either an KRC protein or a molecule in a signal transduction pathway involving KRC, or a target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated protein or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which are reactive with protein or target molecules but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells of the plate, and unbound target or KRC protein is trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the

GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with KRC or a molecule in a signal transduction pathway involving KRC or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the KRC protein or target molecule.

C. Assays Using Knock-Out Cells

In another embodiment, the invention provides methods for identifying compounds that modulate a biological effect of KRC or a molecule in a signal transduction pathway involving KRC using cells deficient in KRC (or e.g., Jun, TRAF, GATA3, SMAD, Runx2). As described in the Examples, inhibition of KRC activity (*e.g.*, by disruption of the KRC gene) in cells results, e.g., in a deficiency of IL-2 production, impaired Th2 cell development, and/or impaired TGFβR signaling. Thus, cells deficient in KRC or a molecule in a signal transduction pathway involving KRC can be used identify agents that modulate a biological response regulated by KRC by means other than modulating KRC itself (*i.e.,* compounds that "rescue" the KRC deficient phenotype). Alternatively, a "conditional knock-out" system, in which the gene is rendered non-functional in a conditional manner, can be used to create deficient cells for use in screening assays. For example, a tetracycline-regulated system for conditional disruption of a gene as described in WO 94/29442 and U.S. Patent No. 5,650,298 can be used to create cells, or animals from which cells can be isolated, be rendered deficient in KRC(or a molecule in a signal transduction pathway involving KRC e.g., Jun, TRAF, GATA3, SMAD, Runx2) in a controlled manner through modulation of the tetracycline concentration in contact with the cells. Specific cell types, e.g., lymphoid cells (*e.g.*, thymic, splenic and/or lymph node cells) or purified cells such as T cells, B cells, osteoblasts, osteoclasts, from such animals can be used in screening assays. In one embodiment, the entire 5.4 kB exon 2 of KRC can be replaced, e.g., with a neomycin cassette, resulting in an allele that produces no KRC protein. This embodiment is described in the appended examples.

In the screening method, cells deficient in KRC or a molecule in a signal transduction pathway involving KRC can be contacted with a test compound and a biological response regulated by KRC or a molecule in a signal transduction pathway involving KRC can be monitored. Modulation of the response in cells deficient in KRC or a molecule in a signal transduction pathway involving KRC (as compared to an

appropriate control such as, for example, untreated cells or cells treated with a control agent) identifies a test compound as a modulator of the KRC regulated response.

In one embodiment, the test compound is administered directly to a non-human knock out animal, preferably a mouse (*e.g.*, a mouse in which the KRC gene or a gene in a signal transduction pathway involving KRC is conditionally disrupted by means described above, or a chimeric mouse in which the lymphoid organs are deficient in KRC or a molecule in a signal transduction pathway involving KRC as described above), to identify a test compound that modulates the *in vivo* responses of cells deficient in KRC. In another embodiment, cells deficient in KRC are isolated from the non-human KRC deficient animal or a molecule in a signal transduction pathway involving KRC deficient animal, and contacted with the test compound *ex vivo* to identify a test compound that modulates a response regulated by KRC in the cells

Cells deficient in KRC or a molecule in a signal transduction pathway involving KRC can be obtained from a non-human animals created to be deficient in KRC or a molecule in a signal transduction pathway involving KRC Preferred non-human animals include monkeys, dogs, cats, mice, rats, cows, horses, goats and sheep. In preferred embodiments, the deficient animal is a mouse. Mice deficient in KRC or a molecule in a signal transduction pathway involving KRC can be made using methods known in the art. One example of such a method and the resulting KRC heterozygous and homozygous animals is described in the appended examples. Non-human animals deficient in a particular gene product typically are created by homologous recombination. In an exemplary embodiment, a vector is prepared which contains at least a portion of the gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the endogenous KRC. The gene preferably is a mouse gene. For example, a mouse KRC gene can be isolated from a mouse genomic DNA library using the mouse KRC cDNA as a probe. The mouse KRC gene then can be used to construct a homologous recombination vector suitable for modulating an endogenous KRC gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector).

Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous KRC protein). In the homologous recombination vector, the altered portion of the gene is flanked at its 5' and 3' ends by additional nucleic acid of the gene to allow for homologous recombination to occur between the exogenous gene carried by the vector and an endogenous gene in an embryonic stem cell. The additional flanking nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced gene has homologously recombined with the endogenous gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In one embodiment of the screening assay, compounds tested for their ability to modulate a biological response regulated by KRC or a molecule in a signal transduction pathway involving KRC are contacted with deficient cells by administering the test compound to a non-human deficient animal *in vivo* and evaluating the effect of the test compound on the response in the animal.

The test compound can be administered to a non-knock out animal as a pharmaceutical composition. Such compositions typically comprise the test compound

and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal compounds, isotonic and absorption delaying compounds, and the like, compatible with pharmaceutical administration. The use of such media and compounds for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or compound is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions. Pharmaceutical compositions are described in more detail below.

In another embodiment, compounds that modulate a biological response regulated by KRC or a signal transduction pathway involving KRC are identified by contacting cells deficient in KRC *ex vivo* with one or more test compounds, and determining the effect of the test compound on a read-out. In one embodiment, KRC deficient cells contacted with a test compound *ex vivo* can be readministered to a subject.

For practicing the screening method *ex vivo*, cells deficient, e.g., in KRC, Jun, TRAF, GATA3, SMAD, and/or Runx, can be isolated from a non-human deficient animal or embryo by standard methods and incubated (*i.e.*, cultured) *in vitro* with a test compound. Cells (*e.g.*, T cells, B cells, and/or osteoblasts) can be isolated from e.g., KRC, Jun, TRAF, GATA3, SMAD, and/or Runx, deficient animals by standard techniques. In another embodiment, the cells are isolated form animals deficient in one or more of KRC, Jun, TRAF, GATA3, SMAD, and/or Runx.

In another embodiment, cells deficient in more than one member of a signal transduction pathway involving KRC can be used in the subject assays.

Following contact of the deficient cells with a test compound (either *ex vivo* or *in vivo*), the effect of the test compound on the biological response regulated by KRC or a molecule in a signal transduction pathway involving KRC can be determined by any one of a variety of suitable methods, such as those set forth herein, *e.g.*, including light microscopic analysis of the cells, histochemical analysis of the cells, production of proteins, induction of certain genes, e.g., cytokine gene, such as IL-2, degradation of certain proteins, *e.g.*, ubiquitination of certain proteins, as described herein.

D. Test Compounds

A variety of test compounds can be evaluated using the screening assays described herein. The term "test compound" includes any reagent or test agent which is employed in the assays of the invention and assayed for its ability to influence the expression and/or activity of KRC or a molecule in a signal transduction pathway involving KRC. More than one compound, *e.g.*, a plurality of compounds, can be tested at the same time for their ability to modulate the expression and/or activity of, e.g., KRC in a screening assay. The term "screening assay" preferably refers to assays which test the ability of a plurality of compounds to influence the readout of choice rather than to tests which test the ability of one compound to influence a readout. Preferably, the subject assays identify compounds not previously known to have the effect that is being screened for. In one embodiment, high throughput screening can be used to assay for the activity of a compound.

In certain embodiments, the compounds to be tested can be derived from libraries (*i.e.*, are members of a library of compounds). While the use of libraries of peptides is well established in the art, new techniques have been developed which have allowed the production of mixtures of other compounds, such as benzodiazepines (Bunin *et al.* (1992). *J. Am. Chem. Soc.* 114:10987; DeWitt *et al.* (1993). *Proc. Natl. Acad. Sci. USA* 90:6909) peptoids (Zuckermann. (1994). *J. Med. Chem.* 37:2678) oligocarbamates (Cho *et al.* (1993). *Science.* 261:1303-), and hydantoins (DeWitt *et al. supra*). An approach for the synthesis of molecular libraries of small organic molecules with a diversity of 104-105 as been described (Carell *et al.* (1994). *Angew. Chem. Int. Ed. Engl.* 33:2059- ; Carell *et al.* (1994) Angew. *Chem. Int. Ed. Engl.* 33:2061-).

The compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the 'one-bead one-compound' library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145). Other exemplary methods for the synthesis of molecular libraries can be found in the art, for example in:

Erb *et al.* (1994). *Proc. Natl. Acad. Sci. USA* 91:11422- ; Horwell *et al.* (1996) *Immunopharmacology* 33:68- ; and in Gallop *et al.* (1994); *J. Med. Chem.* 37:1233-.

Libraries of compounds can be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); In still another embodiment, the combinatorial polypeptides are produced from a cDNA library.

Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries.

Candidate/test compounds include, for example, 1) peptides such as soluble peptides, including Ig-tailed fusion peptides and members of random peptide libraries (see, *e.g.*, Lam, K.S. *et al.* (1991) *Nature* 354:82-84; Houghten, R. *et al.* (1991) *Nature* 354:84-86) and combinatorial chemistry-derived molecular libraries made of D- and/or L- configuration amino acids; 2) phosphopeptides (*e.g.*, members of random and partially degenerate, directed phosphopeptide libraries, see, *e.g.*, Songyang, Z. *et al.* (1993) *Cell* 72:767-778); 3) antibodies (*e.g.*, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, and single chain antibodies as well as Fab, $F(ab')_2$, Fab expression library fragments, and epitope-binding fragments of antibodies); 4) small organic and inorganic molecules (*e.g.*, molecules obtained from combinatorial and natural product libraries); 5) enzymes (*e.g.*, endoribonucleases, hydrolases, nucleases, proteases, synthatases, isomerases, polymerases, kinases, phosphatases, oxido-reductases and ATPases), and 6) mutant forms of KRC (*e.g.*, dominant negative mutant forms of the molecule).

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The

biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds can be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or phage (Scott and Smith (1990) *Science* 249:386-390; Devlin (1990) *Science* 249:404-406; Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici (1991) *J. Mol. Biol.* 222:301-310; Ladner *supra.*).

Compounds identified in the subject screening assays can be used in methods of modulating one or more of the biological responses regulated by KRC. It will be understood that it may be desirable to formulate such compound(s) as pharmaceutical compositions (described supra) prior to contacting them with cells.

Once a test compound is identified that directly or indirectly modulates, e.g., KRC expression or activity, or a molecule in a signal transduction pathway involving KRC, by one of the variety of methods described hereinbefore, the selected test compound (or "compound of interest") can then be further evaluated for its effect on cells, for example by contacting the compound of interest with cells either *in vivo* (*e.g.*, by administering the compound of interest to a subject) or *ex vivo* (*e.g.*, by isolating cells from the subject and contacting the isolated cells with the compound of interest or, alternatively, by contacting the compound of interest with a cell line) and determining the effect of the compound of interest on the cells, as compared to an appropriate control (such as untreated cells or cells treated with a control compound, or carrier, that does not modulate the biological response).

The instant invention also pertains to compounds identified in the subject screening assays.

VI. Kits of the Invention

Another aspect of the invention pertains to kits for carrying out the screening assays, modulatory methods or diagnostic assays of the invention. For example, a kit for carrying out a screening assay of the invention can include an indicator composition comprising KRC or a molecule in a signal transduction pathway involving KRC, means for measuring a readout (*e.g.*, protein secretion) and instructions for using the kit to identify modulators of biological effects of KRC. In another embodiment, a kit for carrying out a screening assay of the invention can include cells deficient in KRC or a molecule in a signal transduction pathway involving KRC, means for measuring the readout and instructions for using the kit to identify modulators of a biological effect of KRC.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis *et al.* U.S. Patent NO: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu *et al.* eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents, and published patent applications cited throughout this application, as well as the figures and the sequence listing, are hereby incorporated by reference.

EXAMPLES

The following materials and methods were used throughout the Examples:

Cell Lines, Plasmids and Stable and Transient Transfection Assays

The human embryonic kidney cell line HEK293, the NIH/3T3 fibroblast cells and the macrophage cell line RAW were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HEK293 cells (4 X 510 5 per well) were seeded in 6 well plates, and 12 h later cells were transfected with EFECTENE™ (Qiagen) with 25 ng of a 2XNFκB-luciferase (Luc) reporter gene plasmid and 0.5 μg of the indicated TRAF and KRC expression vectors. Total amounts of transfected DNA were kept constant by supplementing with control empty expression vector plasmids as needed. Cell extracts were prepared 24 h after transfection, and reporter gene activity was determined via the luciferase assay system (PROMEGA). PRSV-βGal vector (50 ng) was used to normalize for transfection efficiency by measuring β galactosidase activity using the Galacton-PLUS substrate system (TROPIX, Inc.). Whenever indicated, the cells were treated for 4 hours with TNFα or IL-1 (10ng/ml). To generate stable transfectants, EFECTENE™ mediated transfection of the RAW cell line was performed and clones were selected and maintained in complete medium supplemented with G418 (2 mg/ml).

Yeast Two Hybrid Screen

The yeast strain EGY48, containing the reporter genes for LEU and β–galactosidase activity under the control of an upstream LexA-binding site was used as a host for the two hybrid screen. The KRC fragment from amino acid 204 to 1055 (KRC tr) ~~(*Figure 2*(A))~~ was fused in frame to the LexA DNA binding domain and a yeast strain expressing the LexA-KRC tr fusion protein was transfected with a mouse Th1

clone cDNA library (Szabo, *et al.*) fused to the GAL4 transcriptional activation domain. Transformants were plated on agar selection media lacking uracil, tryptophan, leucine and histidine. The resulting colonies were isolated and retested for growth in Leu^- plates and for β galactosidase activity. Plasmid DNA was purified from colonies that were $Leu^+\beta gal^+$ and used for retransformation of a yeast strain expressing a heterologous bait to determine the specificity of interaction.

Northern Blot Analysis

Total RNA was isolated from transfected RAW macrophage cells using TRIZOL™ reagent (Gibco/BRL) and 15 μg of each sample separated on 0.8% agarose 6% formaldehyde gels, transferred onto GeneScreen™ membrane (NEN) in 20X SSC overnight and covalently bound using a UV Stratalinker™ (Stratagene). Hybridization of blots was carried out at 42^oC as described (Hodge, *et al.*) using the radiolabeled TNFα, KRC (5850-6210) and HPRT probes prepared with the Random primer kit (Boehringer Mannheim).

Western Blot Analysis

Effectene™ mediated transfections into 293T cells were performed. To prepare cell extracts, cells were washed twice with PBS and lysed for 10 minutes on ice in 1 ml Triton lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 2mM DTT and complete protease inhibitor mixture (Roche Molecular Biochemicals), and the lysates were cleared by centrifugation for 10 min at 14 000 rpm. The cell lysates were precleared with 30 μl of protein A/G-Sepharose beads and then incubated for 4h with 25 μl of anti-MYC antibody directly conjugated to sepharose beads. The immunoprecipitates were then washed 5 times with the lysis buffer, resuspended in SDS sample buffer, and heated at 95^0 C for 5 min. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitocellulose membrane (Schleicher & Schuell) and western blotting performed by probing with primary antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

In vitro Kinase Assay

Anti-HA or anti-FLAG immunoprecipitates were used for immune complex kinase assays that were performed at 30^0 C for 30 min with 1 μg of substrate, 10 μCi of γ^{32} P ATP, and 10 μM ATP in 30 μl of kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl2, 25 mM β-glycerophosphate, 50 μm NA3VO4, and 50 μm DTT). The substrate was GST-c-JUN.

Apoptosis Assay

β–galactosidase cotransfection assays for determination of cell death were performed as described (Hsu, *et al.*). Transfected NIH 3T3 cells were washed with PBS, fixed in PBS containing 3% paraformaldehyde for 10 min at 4^0 C, and washed with PBS. Fixed cells were stained overnight with XGal. The number of blue-stained cells was determined microscopically. The average number from one representative experiment of three is shown.

Luciferase Assays

For each transfection, 5 x 10^6 Jurkat cells were incubated with either IL2-Luc, NFAT/AP1-Luc or AP1-Luc reporter DNA together with pEF vector or pEF-KRC and CMV-βGAL as normalization control in 0.4 ml of RPMI and transfected by electroporation (260 v, 975uF). Transfected cells were cultured at 37° C for 20 h in RPMI 1640 medium (Gibco BRL) supplemented with 10 % fetal bovine serum. Transfected cells were stimulated with PMA (50ng/ml) and ionomycin (2uM) for 6 hours prior to luciferase (Promega) and β-galactosidase assays (Galacton-PLUS substrate system, TROPIX, Inc).

Reverse Transcription-PCR

Total RNA was isolated from T cells using TRIZOL™ reagent (Gibco/BRL). One (1) μg of total RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad). PCR was performed with 2uM of each primer (listed below) and 2.5 units of Platinum High fidelity enzyme (Invitrogen) according to the manufacturer.

IL2F 5'CAAGAATCCAAACTCACCAG3' (SEQ ID NO:3),

IL2R 5'TAGCAACCATACATTCAACAA3' (SEQ ID NO:4)

KRCF 5'CTCCAATACAGAATTCAAGGGC3' (SEQ ID NO:5),

KRCR5'TTTAGGTTGGCCAGTGTGTGTG (SEQ ID NO:6)

Jurkat Cell Activation With Raji B Lymphoma Cells and Staphylococcal Enteroxin E (SEE)

Jurkat cells were transfected by electroporation and incubated for 20 h at 37^0 C before stimulation for 8 h with the Raji B cell line and Staphylococcal Enteroxin E (SEE) using Raji cells (1:1 with Jurkat cells) and SEE (200 ng/ml).

Pull Down Assays

In vitro translated c-Jun (35^S methionine labeled) and His-KRCtr were incubated for 2h at 4^0 C in binding buffer (PBS/0.25 % Nonidet p-40/1mM PMSF/0.25 mM DTT), incubated for 2 hours with the anti-HIS antibody (Santa Cruz), 30 µl of protein A/G sepharose added and the reaction incubated at 4^0 C for an additional 2h. The immunoprecipitates were then washed five times with the binding buffer, resuspended in SDS sample buffer, and heated at 95^0 C for 5 min.

Retroviral Gene Transduction

Activated $CD4^+$T cells were transduced by RV, RV-KRC or RV-ZAS2 as described previously (Szabo, S.J., *et al.* (2000) *Cell* 100:655-669).

Generation of KRC-Deficient Mice and Subsequent T Cell Stimulation

ES cells were generated in which the entire 5.4 kB exon 2 of KRC was replaced by a neomycin cassette resulting in an allele that produces no KRC protein. KRC +/- ES cells transmitted the disrupted allele to 129/B6 offspring. Heterozygous pups were backcrossed to wild type B6 mice. Mice analyzed were progeny of intercrosses between heterozygous F3 generation backcrossed 129/B6 mice. CD4+ T cells were purified by positive selection from spleen and lymph nodes of 6-8 week old male KRC +/+ and KRC -/- littermates using magnetic beads according to the instructions of the manufacturer (Miltenyi Biotec). Cells were stimulated at 10^6 cells/mL with plate-bound anti-CD3 (1.0 µg/mL) plus anti-CD28 (0.5 µg/mL). Twenty-four hours later, supernatants were collected and analyzed for IL-2 levels by ELISA. Additionally, cells were stimulated for 72 hours in the presence of 200 U/mL human IL-2, and supernatants were collected and analyzed for IFNγ levels by ELISA.

EXAMPLE 1: INTERACTION OF KRC WITH TRAF FAMILY MEMBERS IN YEAST

(A) In this example, a yeast two-hybrid interaction trap was used to select a T cell cDNA library for sequences encoding polypeptides that specifically interacted with a KRC-LexA fusion protein. As bait KRC sequences encoding amino acids 204 to 1055 (KRC tr) were used which include the third zinc finger domain, one of the three acidic domains and the putative NLS sequence, expressed in the pEG202 vector. One class of interactors encoding a fusion protein with apparently high affinity for the KRC-LexA bait as exhibited by high level of β-galactosidase activity and ability to confer leucine prototrophy was isolated and upon sequencing proved to be the C-terminal segment of TRAF1. The interaction with TRAF1 was specific since no interaction was detected with control plasmids that encode KRC, c-Maf or relA fusion proteins or with the control vector alone

(B) The ability of TRAF proteins to interact specifically with KRC *in vivo* was tested in mammalian cells. KRC sequences 204-1055 were subcloned into a mammalian expression vector which fuses the coding region to an N-terminal epitope tag from a myc peptide, and the expression of the protein confirmed by direct western blot analysis with anti-MYC antibody. This tagged construct was then cotransfected with TRAF-FLAG-tagged expression plasmids into 293T cells and lysates prepared for immunoprecipitation with an anti-MYC antibody. A STAT4-FLAG-tagged expression construct was used as negative control.

Western blot analysis of these samples using an anti-FLAG-specific monoclonal antibody (mAb) demonstrated that the anti-MYC antibody coimmunoprecipitated all six FLAG-tagged TRAFs, but not the STAT4 control protein. Finally, the deletion of the ring finger of TRAF2 (TRAF2 DN) did not alter its interaction with KRC, consistent with our isolation of TRAF1, which lacks the RING finger, in the yeast two hybrid interaction trap screen. These results demonstrate that KRC does interact with all TRAF family members and that this interaction is likely occurring through the TRAF C domain.

~~(C)~~ Coimmunoprecipitation assays in the presence of more stringent, higher salt conditions were performed. When_300mM rather than 137mM NaCl was used, TRAF5 was not able to coimmunoprecipitate with KRC, and the amount of TRAFs 3, 4 and 6 that could be immunoprecipitated was reduced. The TRAF-C domain of TRAF1 and TRAF2 share 70 % identity but share less than 43% identity with TRAF5 and TRAF(

D) To further explore if KRC interacted with a higher affinity with TRAF1 and TRAF2 and with lower affinity with the other TRAF members, we tested the association of endogenous rather than overexpressed TRAFs with ectopically expressed KRC. 293T cells (which lack TRAF1) were transfected with plasmids encoding MYC-tagged KRC or empty vector and 24 hours after transfection cells were lysed. Lysates from 293T cells were incubated with anti-MYC antibody to precipitate KRC.

Subsequent Western blotting with anti-TRAF2, anti-TRAF5 or anti-TRAF6 mAbs showed that only endogenous TRAF2 was able to interact with over-expressed KRC. The bands observed in the TRAFs 5 and 6 coimmunoprecipitants are non-specific Furthermore, treatment of 293T cells with TNF or IL-1 to induce TRAF activity did not affect the strength of the interaction between TRAF2 and ectopically expressed KRC).

Taken together, these data demonstrate that KRC interacts with TRAF family members, that this interaction occurs through the TRAF-C domain, and that KRC interacts with higher affinity with TRAF2 than with TRAF5 and TRAF6. This result is consistent with the higher sequence conservation between the TRAF domain of TRAF1 and TRAF2 than between the other TRAF family members.

EXAMPLE 2: KRC PREVENTS TRAF DEPENDENT NFκB ACTIVATION

In this example, the effect of KRC overexpression on TRAF2, TRAF5 and TRAF6-induced NFκB dependent gene expression using transfection assays in 293T human embryonic kidney cells was tested. The results show that overexpression of both the full-length KRC and the KRC 204-1055 (KRC truncated, tr) in the absence of exogenous TRAFs blocked NFκB-dependent transactivation in a manner comparable in strength to the inhibition observed with a dominant negative form of TRAF2. The results also show that both the KRC tr and the full length KRC blocked TRAF2-induced NFκB activation while NFκB activation induced by TRAF5 and TRAF6 were substantially but not completely affected.

EXAMPLE 3: ANTISENSE AND DOMINANT NEGATIVE KRC INCREASE CYTOKINE DRIVEN NFκB TRANSACTIVATION WHILE SENSE KRC IS INHIBITORY.

(A) In this example, whether KRC overexpression affects TNFα-induced NFκB transactivation in 293 cells was tested. Overexpression of KRC or KRC tr in 293 cells strongly inhibited TNFα-induced NFκB activation to a level comparable with the

TRAF2 DN effect in the presence of TNFα. These data are consistent with the demonstrated effect of TRAF2 on NFκB-dependent gene activation in certain cell types, *e.g.*, B cells, as shown in TRAF2-deficient mice (Yeh, *et al.*).

(B) To manipulate the endogenous KRC, an antisense KRC construct (H10AS) and a dominant negative construct expressing only the ZAS2 domain of KRC (ZAS2) was used. Both the antisense and the ZAS2 expressing constructs greatly enhanced transactivation of the NFκB reporter upon induction with TNFα. The same results were obtained with the antisense KRC and dominant negative KRC when NFκB-dependent transactivation was driven by exogenous TRAF2 overexpression. These results demonstrate that KRC under normal conditions behaves as a negative regulator of TRAF2-mediated NFκB activation.

EXAMPLE 4: IKKβ OVEREXPRESSION OVERCOMES KRC INHIBITION OF NFκB-DEPENDENT TRANSACTIVATION

In this example, whether KRC affected NFκB-driven gene activation by interfering with upstream events was tested. Full-length KRC or KRC tr, and as a control, the TRAF2 DN mutant, were overexpressed in 293 cells in the absence or presence of ectopic IKKβ (IκB kinase) and the effect on NFκB-mediated transactivation determined. The activation of IKKβ is a key step in the nuclear translocation of the transcription factor NF-κB. IKK is a complex composed of three subunits: IKKα, IKKβ, and IKKγ (also called NEMO). In response to the proinflammatory cytokine tumor necrosis factor (TNF), IKK is activated after being recruited to the TNF receptor 1 (TNF-R1) complex via TNF receptor-associated factor 2 (TRAF2). Overexpression of IKKβ overcomes the inhibitory effect of both KRC and KRC tr in a manner comparable to its effect on TRAF2 DN. Since IKK activation is downstream of TRAF activation, these results demonstrate that the effect of KRC on NFκB-driven gene expression is due to its ability to interact with TRAFs rather than to competition with NFκB for binding to DNA.

EXAMPLE 5: KRC INCREASES TNFα-INDUCED APOPTOSIS

In this example, whether KRC is involved in the apoptotic process was tested. KRC was overexpressed in 3T3 cells apoptosis was measured by counting β-galactosidase positive (live) cells. As previously described for HeLa cells, these results

demonstrate that in 3T3 cells apoptosis can be induced when either IκB DN or TRAF2 DN are overexpressed in the presence of TNFα, but cannot be induced by TNFα alone (Hsu, *et al.*; Hsu, *et al.*; Liu, *et al.*). KRC overexpression resulted in an increase in TNF mediated cytotoxicity equivalent to that observed with overexpression of IκB or TRAF2 DN. The same effect was observed with the KRC tr construct indicating that KRC likely sensitizes cells to TNFα-induced death by inhibiting NFκB induction, most probably through its effect on blocking TRAF2 function. Collectively, these results demonstrate that upon TNF receptor activation, the NFkB, TRAF1, TRAF2, c-IAP-1 and c-IAP-2 pathways operate as a positive feedback system to amplify the survival signal to protect cells from TNF-induced injury. The interaction of KRC with TRAF2, and possibly with TRAF1 in other cell types, acts to inhibit TRAF activity thereby balance between pro-apoptotic and anti-apoptotic stimuli.

EXAMPLE 6: KRC PREVENTS TRAF2 AND TNFα-DEPENDENT JNK ACTIVATION

In this example, whether KRC could block TRAF2 dependent JNK activation was tested. The KRC 204-1055 tr construct, full length KRC, ZAS2 expressing construct and the antisense KRC were cotransfected into 293 cells together with TRAF2, and JNK activity measured 24 hours after transfection. Both the KRC tr and the full length KRC blocked TRAF2-dependent JNK activation. Full length KRC blocked JNK activation only partially, likely due to the approximately 10 fold lower expression of this construct as compared to KRC tr. The results also show a dramatic increase of TRAF2 dependent JNK activation with expression of both the antisense KRC as well with the dominant negative ZAS2 expressing construct.

The same results were obtained when JNK activation was induced by treatment with TNFα. A careful time course of JNK activation was performed, mediated by TNFα in the presence of antisense KRC, which revealed sustained JNK activation as compared to control vector alone. These results demonstrate that KRC negatively modulates JNK activation by inhibiting TRAF2 function. The immediate target of TRAF2 in TNF-induced JNK/SAPK activation may be the MAP3 kinase ASK1 or members of the GCK family of kinases.

EXAMPLE 7: KRC IS A NEGATIVE REGULATOR OF ENDOGENOUS TNFα EXPRESSION

In this example, whether KRC can modulate the expression of endogenous TNFα was tested. Overexpressed KRC or dominant negative KRC was transfected in the RAW macrophage cell line and levels of TNFα in a panel of transfectant clones were analyzed. RAW transfectants stably overexpressing KRC displayed a substantial decrease of baseline TNFα mRNA transcripts when compared to control vector transfected RAW cells while RAW transfectants expressing the dominant negative version had substantial increase in TNFα expression. These results demonstrate that KRC acts to inhibit the transcription of the TNFα proinflammatory cytokine and that this may occur both through its inhibition of NFκB and JNK signaling pathways.

EXAMPLE 8: KRC TRANSLOCATES FROM CYTOSOL TO NUCLEUS UPON CELL ATTACHMENT

In this example, how KRC (originally decried as a nuclear protein) physiologically interacts with the predominantly cytosolic TRAF2 to affect gene activation was tested. A full-length KRC was fused to GFP and its cellular localization upon transfection into 3T3 cells was examined. In 3T3 cells in suspension, KRC was mainly localized to the cytosol while in 3T3 cells that had adhered to the glass slide, KRC was primarily present in the nucleus. These results clearly demonstrate that KRC can reside in the cytosol where it can interact with TRAF2. It should be noted that TRAF2 has recently been described to translocate from cytosol to nucleus as well (Min, *et al*, 1998). Thus KRC and TRAF2 may well interact in both subcellular compartments.

EXAMPLE 9: KRC EXPRESSION IS MAINTAINED IN TH1 CELLS

In this example, KRC expression in primary T cells was measured. RT-PCR analysis of KRC expression in primary T cells was performed. KRC expression was measured at 24 hours and 72 hours. The results demonstrate that KRC expression is rapidly lost in Th2 cells at 72 hours whereas KRC expression in Th1 cells is maintained at 72 hours.

EXAMPLE 10: KRC ACTIVATES T CELLS

In this example, KRC was transfected into Jurkat T cells and CD69 expression was measured by FACS analysis. The results show that KRC overexpression increases expression of CD69 (a T cell activation marker) in Jurkat T cells.

EXAMPLE 11: KRC INCREASES IL-2 GENE TRANSCRIPTION IN THE PRESENCE OF PMA/IONOMYCIN

This example shows that KRC increases IL-2 gene transcription in the presence of PMA/Ionomycin. This increase in IL-2 transcription occurs primarily through activating AP-1 with no contribution from NFAT. IL-2 promoter transactivation by KRC in Jurkat T cells activated by PMA/Ionomycin. Transactivation of a composite NFAT-AP1 reporter by KRC. Transactivation of an AP-1 reporter by KRC.

EXAMPLE 12: KRC INCREASES IL-2 GENE TRANSCRIPTION IN THE PRESENCE OF B CELL ANTIGEN PRESENTING CELLS

In this example, the results demonstrate that KRC increases IL-2 gene transcription in the presence of B cell antigen presenting cells and superantigen SEE and does so primarily through activating AP-1 with no contribution from NFAT. IL-2 promoter transactivation by KRC in Jurkat T cells activated by the Raji B cell APC line and the superantigen.

EXAMPLE 13: KRC OVEREXPRESSION INCREASES ENDOGENOUS IL-2 PRODUCTION WHILE KRC LOSS DECREASES ENDOGENOUS IL-2 PRODUCTION

In this example, increased IL-2 production in Jurkat T cells stably expressing KRC was measured by ELISA. IL-2 promoter activation requires antigen receptor engagement plus an accessory signal usually supplied by an antigen presenting cell (Jain, J., *et al.* (1995) *Curr. Biol.* 7:333-342). Agents that bypass these receptors, such as PMA and ionomycin, can mimic T cell activation in the human T cell lymphoma Jurkat. To assess the function of KRC in T cells, Jurkat cells, which express barely detectable levels of endogenous KRC protein by Western blot analysis, were stably transfected with a plasmid encoding full-length KRC (pEF-KRC) or with vector only control (pEF). G418 drug- resistant Jurkat clones were expanded and analyzed for IL-2 secretion following activation. Clones stably expressing KRC showed clear increases in KRC protein levels, as detected by Western blotting All clones expressing pEF-KRC produced substantially greater amounts of IL-2 upon PMA and ionomycin treatment than activated Jurkat clones transfected with the control vector. KRC overexpression alone was not sufficient to induce IL-2 secretion, as no IL-2 was detected in the culture supernatants of unstimulated KRC-overexpressing clones These results suggested that KRC is able to boost IL-2 secretion in concert with signals emanating from the TCR.

Although the Jurkat model has proved valuable to dissect pathways of T cell signaling, certain observations made in Jurkat cells are irreproducible in primary T cells (Dumitru, C.D. *et al.* (2000) *Cell* 103:1071-1083; Weiss, L., *et al.* (2000) *J. Exp. Med.* 191: 139-145). Therefore, the effects of KRC overexpression were studied in primary CD4+T cells as well as in the Jurkat line using a retroviral delivery system to express KRC in primary CD4+T cells. Bicistronic retroviral vectors encoding full-length KRC and control GFP were generated. The KRC ZAS2 domain was previously shown to act as a dominant negative in the context of KRC mediated inhibition of TNF-induced NF-κB activation (Oukka, M., *et al.* (2002) *Mol. Cell* 9:121-131). Purified CD4+T cells were infected with these retroviruses 36 hours after primary activation with both anti-CD3 and anti-CD28, and sorted by flow cytometry for GFP expression 24 hours after infection. The ability of each population to produce IL-2 following subsequent activation by anti CD3 or anti CD3 plus CD28 was measured at 24 hours post-stimulation. CD4 cells transduced with full-length KRC produced higher amounts (approximately 3 to 4 fold increase) of IL-2 than CD4 cells infected with the GFP control retrovirus. Furthermore, CD4 cells transduced with the dominant negative KRC ZAS2 domain construct produced significantly less IL-2 than both the full-length KRC and GFP control transduced cells. These data are consistent with the notion that the ZAS2 domain interferes with endogenous KRC activity in T cells to prevent optimal expression of IL-2.

EXAMPLE 14: KRC TRANSACTIVATION OF AP-1 DEPENDS ON RAS, RAF AND PKC-THETA

In this example, the results demonstrate that KRC transactivation of AP-1 response element depends on Ras, Raf and PKC-theta signaling molecules. KRC transactivation of the AP-1 reporter is blocked by dominant negative Ras and Raf. KRC transactivation of the AP-1 reporter is blocked by dominant negative PKC-theta and by the specific PKC-theta inhibitor Rottlerin.

EXAMPLE 15: KRC CONTROLS IL-2 EXPRESSION

In this example, the results demonstrate that KRC controls IL-2 expression. RT-PCR of KRC transfected Jurkat clones was performed. The results show increased IL-2 expression upon KRC transfection.

EXAMPLE 16: KRC INCREASES ACTIN POLYMERIZATION

In this example, the results demonstrate that KRC increases actin polymerization. Immunofluorescence of F-actin upon KRC overexpression in Jurkat T cells was performed. The results show the reorganization of F-actin filaments in KRC transfected Jurkat T cells.

5

EXAMPLE 17: KRC EXPRESSION INCREASES IN $CD4^+$ CELLS UPON ACTIVATION

In this example, the results demonstrate that KRC expression increases in $CD4^+$ cells upon activation with anti-CD3 ((2.0 μg/mL)/anti-CD28 (1.0 μg/mL) antibodies. RT-PCR analysis demonstrates that KRC expression was induced with very rapid kinetics (within 20 minutes) in $CD4^+$ T cells upon activation and increased levels of KRC transcripts were observed throughout the duration of primary CD3/CD28 stimulation, up to 48 hours.

EXAMPLE 18: KRC OVEREXPRESSION INCREASES WHILE KRC LOSS DECREASES ENDOGENOUS IL-2 PRODUCTION IN BOTH TRANSFORMED AND PRIMARY T CELLS

IL-2 promoter activation requires antigen receptor engagement plus an accessory signal usually supplied by an antigen presenting cell (Jain, J., C. Loh, and A. Rao. 1995. 7:333-342.). Agents that bypass these receptors, such as PMA and ionomycin, can mimic T cell activation in the human T cell lymphoma Jurkat. To assess the function of KRC in T cells, Jurkat cells, which express barely detectable levels of endogenous KRC protein by Western blot analysis, were stably transfected with a plasmid encoding full-length KRC (pEF-KRC) or with vector only control (pEF). G418 drug- resistant Jurkat clones were expanded and analyzed for IL-2 secretion following activation. Clones stably expressing KRC showed clear increases in KRC protein levels, as detected by Western blotting. All clones expressing pEF-KRC produced substantially greater amounts of IL-2 upon PMA and ionomycin treatment than activated Jurkat clones transfected with the control vector. KRC overexpression alone was not sufficient to induce IL-2 secretion, as no IL-2 was detected in the culture supernatants of unstimulated KRC-overexpressing clones. These results suggested that KRC is able to boost IL-2 secretion in concert with signals emanating from the TCR.

Although the Jurkat model has proved valuable to dissect pathways of T cell signaling, certain observations made in Jurkat cells are irreproducible in primary T cells Although the Jurkat model has proved valuable to dissect pathways of T cell activation and signaling, some observations made in Jurkat cells have not been reproduced in primary T cells(Dumitru, C.D., J.D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J.-H. Lin, C. Patriotis, N.A. Jenkins, N.G. Copeland, G. Kollias, and P.N. Tsichlis. 2000. TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103:1071-1083, Weiss, L. et al. 2000. *J Exp Med* 191: 139-145). Therefore, the effects of KRC overexpression in primary CD4 T cells as well as in the Jurkat line were studied using a retroviral delivery system was used to express KRC in primary CD4 T cells. Bicistronic retroviral vectors encoding full-length KRC were generated, the KRC ZAS2 domain which we have previously shown acts as a dominant negative in the context of KRC mediated inhibition of TNF-induced NF-κB activation (Oukka, .NET al. 2002. *Mol. Cell* 9:121-131), and control GFP. Purified CD4 T cells were infected with these retroviruses 36 hours after primary activation with both anti-CD3 and anti-CD28, and sorted by flow cytometry for GFP expression 24 hours after infection. The ability of each population to produce IL-2 following subsequent activation by anti CD3 or anti CD3 plus CD28 was measured at 24 hours post-stimulation. CD4 cells transduced with full-length KRC produced higher amounts (approximately 3 to 4 fold increase) of IL-2 than CD4 cells infected with the GFP control retrovirus. Furthermore, CD4 cells transduced with the dominant negative KRC ZAS2 domain construct produced significantly less IL-2 than both the full-length KRC and GFP control transduced cells. These data are consistent with the notion that the ZAS2 domain interferes with endogenous KRC activity in T cells to prevent optimal expression of IL-2.

To further analyze the role of KRC in regulating endogenous IL-2 expression, CD4 cells purified from KRC-deficient mice were analyzed. Briefly, lymphoid development in these mice appears normal, with normal numbers of $CD4^+$ T cells isolated from spleen and lymph nodes. Additionally, resting CD4 cells recovered appeared phenotypically normal based on expression of maturation markers such as CD4, CD62L, CD25, CD69 and TCRß. KRC -/- CD4 cells activated *in vitro* for 24 hours by CD3/CD28 stimulation produced 10-fold less IL-2 production was detected

than in CD4 cells from wild type littermates. However, IFNγ production by these cells following 72 hours of primary stimulation in the presence of excess exogenous IL-2 was normal, suggesting that the deficiency of KRC in these cells does not globally inhibit activation-induced cytokine production. Thus, KRC is a positive regulator of IL-2 production both in Jurkat cells and, more importantly, in primary CD4 T cells.

EXAMPLE 19: KRC OVEREXPRESSION INCREASES THE TRANSCRIPTION OF THE IL-2 GENE THROUGH AN AP-1-SITE-DEPENDENT MECHANISM

In this example, the results demonstrate that KRC overexpression increases the transcription of the IL-2 gene through an AP-1-site-dependent mechanism.

The production of IL-2 by T cells is regulated at multiple levels including transcription, mRNA stability and rate of protein secretion (Lindsten, T., *et al.* (1989) *Science* 244:339; Jain, J., *et al.* (1992) *Nature* 356:801-804). In order to define at which stage(s) KRC acts, levels of IL-2 mRNA transcripts were measured by semi-quantitative RT PCR in Jurkat T cells stably transfected with full-length KRC. Jurkat clones over-expressing KRC displayed higher levels of IL-2 transcripts when activated than Jurkat clones transfected with vector control. Next the ability of KRC to directly transactivate a 1.5 kb IL-2 promoter-luciferase reporter in Jurkat cells was tested. Provision of KRC resulted in an approximately 10 fold induction of luciferase activity in Jurkat cells treated with PMA plus ionomycin. Just as KRC overexpression alone did not lead to spontaneous production of endogenous IL-2, no transactivation by KRC was observed in the absence of PMA/ionomycin in these luciferase reporter assays. In order to provide a more physiologic signal to activate Jurkat cells, a model system in which Raji B lymphoma cells act as antigen presenting cells to present staphylococcal enteroxin E (SEE) to Jurkat was utilized. Provision of KRC substantially increased (approximately 10 fold) IL-2 promoter activity in this system. Interestingly, KRC had no effect on IL-2 promoter activity in the absence of Jurkat activation either by PMA/ionomycin or by antigen/APC. These data further suggest that KRC expression alone is not sufficient to induce IL-2 mRNA expression; instead, KRC's ability to enhance IL-2 production relies on endogenous factors found only in activated T cells.

KRC was originally cloned as a transcription factor, however, its effect on gene activation could clearly be ascribed to its function as an adapter protein. Nevertheless,

KRC has been shown to bind both NFκB and RSS target sites *in vitro* and an NFκB site is present in the IL-2 promoter that has been shown to bind the NFκB family member c-Rel (Himes, S.R., *et al.* (1996) *Immunity* 5:479-489). To test whether KRC overexpression leads to enhanced function of a specific site in the IL-2 promoter and to identify the site, Jurkat cells were cotransfected with KRC and various deletion constructs of the IL-2 promoter. In initial experiments, KRC transactivated a luciferase reporter driven by only 200 bp of the IL-2 proximal promoter. The most prominent regulatory sequences in this region are cis elements that bind members of the NFAT, NFκB, and AP-1 transcription factor families (Jain, J., C., *et al.* (1995) *Curr. Biol.* 7:333-342; Ullman, K.S., *et al.* (1993) *Genes & Development* 7:188-196; Rooney, J.W., *et al.* (1995) *Immunity* 2:473-483; Durand, D.B., *et al.* (1987) J. Exp. Med. 165:395-407), although the NFAT and NFκB cis elements have been shown to overlap. Therefore, whether KRC could transactivate a multimerized linked NFAT/AP-1 target site, or individual multimerized NFAT or AP-1 target sites was tested. KRC enhanced PMA/ionomycin-induced transactivation of a multimerized linked NFAT/AP-1 element and the isolated, multimerized AP-1 element but not the NFAT element ~~(*Figure 19(C)*)~~. In contrast to AP-1, the PMA/ionomycin induced activity of NFAT was not further increased by coexpression of KRC. KRC therefore acts at the transcriptional level to increase expression of IL-2 through an AP-1-site-dependent mechanism. Preliminary results show that KRC overexpression enhances, and KRC deficiency decreases, stimulation-induced upregulation of CD69 another AP-1 target gene in T cells (Castellanos, M.C., *et al.* (1997) *J. Immunol.* 159: 5463-5473).

EXAMPLE 20: KRC DOES NOT MODULATE MAPK ACTIVITY

In this example, the results demonstrate that KRC does not modulate MAPK activity. It was unlikely that KRC, a zing finger protein, transactivated the IL-2 promoter through direct binding to the AP-1 element, especially given the observation that KRC was able to enhance AP-1 activity only when Jurkat cells were simultaneously stimulated through the TCR pathway by PMA or antigen/APC. Indeed in EMSA assays using extracts prepared from unstimulated Jurkat cells overexpressing KRC, no binding to a radiolabeled AP-1 site oligonucleotide was detected. Thus, KRC and AP-1 do not bind the same site within the IL-2 promoter to synergistically increase promoter activity.

Additionally, we observed that KRC does not increase AP-1 activity by increasing the expression of c-Jun/c-Fos mRNA

An alternative explanation was that KRC acts upstream to enhance posttranslational modifications of AP-1 that increase its activity. For example, N-terminal phosphorylation of c-Jun or C-terminal phosphorylation of c-Fos have been shown to enhance AP-1 activation downstream of the Ras pathway (Dumitru, C.D., *et al.* (2000) *Cell* 103:1071-1083; Binetruy, B., *et al.* (1991) *Nature* 351:122-127; Deng, T., and M. Karin (1994) *Nature* 371:171-175). Overexpression of a dominant negative Ras blocks TCR-induced AP-1 activity (Rayter, S.I., *et al.* (1992) *Embo J.* 11:4549-4556). More recently, it has been shown that mice deficient in PKC theta show defective TCR induced AP-1 activation, suggesting a role for this kinase in Ras/MAPK/AP-1 activation (Sun, Z., *et al.* (2000) *Nature* 404; Isakov, N., and A. Altman (2002) *Annu. Rev. Immunol.* 20:761-794). Both rottlerin, a PKC theta inhibitor, and overexpression of dominant negative Ras (RasN17) abolished the ability of KRC to enhance AP-1 transactivation following PMA/ionomycin stimulation. These data are consistent with the placement of KRC downstream of the Ras pathway or with a requirement for two distinct, but interconnected signals for IL-2 promoter transactivation. The latter explanation is more likely since KRC can increase AP-1 activation by Ras but cannot activate AP-1 on its own. Thus, KRC activation of AP-1 requires Ras, and KRC can substantially augment AP-1 activation by the Ras pathway.

KRC may enhance AP-1 function indirectly through the modulation of MAPK activity, kinases downstream of Ras that are known to potently stimulate AP-1 function (Binetruy, B., *et al.* (1991) *Nature* 351:122-127; Deng, T., and M. Karin (1994) *Nature* 371:171-175; Murphy, L., *et al.* (2002) *Nat. Cell Biol.* 4: 556-564). In T cells, stimulation via the TCR or with PMA/ionomycin induces the activation of three MAPKs: ERK, p38 and JNK. The activation of these MAPKS is required for AP-1 transcriptional activity. JNK, in particular, has been shown to increase AP-1 transcriptional activity by phosphorylating c-Jun (Arias, J., *et al.* (1994) *Nature* 370:226-229). In initial experiments it was determined that KRC overexpression did not alter levels of transcripts encoding a series of MAP3, MAP2 and MAP kinases as assessed by RNase protection assays (Pharmingen). To test whether KRC had any effect on MAPK activity, a sensitive assay, the PathDetect reporting system, was utilized to evaluate the

effect of KRC on ERK-mediated ELK-1 transactivation and p38-mediated ATF2 transactivation. Jurkat cells were co-transfected with a pGAL4-UAS-LUC reporter and expression plasmids encoding GAL4-Elk1 and GAL4-ATF2 fusion proteins, respectively. KRC was unable to modulate either MAPK or p38 activity in this assay. Co-expression of KRC with HA-ERK1, myc-ERK2, Flag-P38 and Flag-JNK2 was performed and the activity of each kinase was measured using an immunoprecipitation-kinase assay with specific substrates, GST-Elk1, GST-ATF2 and GST-Jun for each MAPK. KRC had no detectable effect on any of the MAPKs in this assay. Therefore, KRC does not increase AP-1 activity through increasing TCR mediated MAPK activity, although it was observed that KRC downregulates TRAF2-mediated JNK activation following TNFα stimulation in macrophage cell lines (Oukka, M., *et al.* (2002) *Mol. Cell* 9:121-131). Since PMA/ionomycin is a very poor inducer of JNK activation in T cells, the possibility that KRC might also downregulate JNK in T cells under different circumstances cannot be ruled out (*e.g.*, CD28 stimulation). However, the ability of KRC to *inhibit* low levels of JNK activity following prolonged CD3/CD28 stimulation of naïve Thp cells is unlikely to account for its ability to dramatically enhance AP-1 function and IL-2 production.

EXAMPLE 21: KRC PHYSICALLY INTERACTS WITH c-Jun AND ACTS AS A TRANSCRIPTIONAL COACTIVATOR

In this example, the results demonstrate that KRC physically interacts with c-Jun and acts as a transcriptional coactivator. It has been demonstrated that KRC interacts with the adapter protein TRAF2 to inhibit both NFκB and JNK/SAPK mediated responses including apoptosis and TNFα cytokine gene expression (Oukka, M., et al. 2002. *Mol. Cell* 9:121-131). To investigate whether KRC might therefore physically associate with c-Jun, expression vectors encoding c-Jun and a truncated myc-tagged version of KRC encoding amino acids 204 to 1055 (KRC tr), which includes the third zinc finger domain, one of the three acidic domains and the putative NLS sequence were overexpressed in the 293T kidney epithelial cell line. Coimmunoprecipitation using a monoclonal anti-myc antibody revealed that KRC physically associated with c-Jun ~~(*Figure 21(A)*)~~. Further, it demonstrated that the region of KRC shown to associate with TRAF2 (aa 204-1055) also interacted with c-Jun. Similar results were obtained in coimmunopreciptations of overexpressed full-length KRC with c-Jun, although the

absolute amounts of c-Jun obtained were less, presumably because the full-length KRC protein is poorly expressed due to its large size. Further mapping of c-Jun to delineate its interaction site with KRC revealed that KRC interacts with c-Jun amino acids 1-224 fused to the DNA binding domain of GAL4, which includes the transactivation domain Further, this association is direct and does not require posttranslational modifications as shown by the interaction of in vitro translated KRC and c-Jun proteins. Finally, it was important to demonstrate that this association occurred under physiologic conditions. Untransfected Jurkat or EL4 T cell lines were stimulated with PMA/ionomycin for 45 minutes, and AP-1 complexes were purified by immunoprecipitating c-Jun. Endogenous KRC is readily detected in these complexes obtained from stimulated cells.

To further investigate the mechanism via which KRC serves as an AP-1 coactivator, AP-1 was activated by overexpressing c-Jun or c-Jun and c-Fos in 293T cells with an AP-1 luciferase reporter. In this system, overexpression of KRC enhances both c-Jun and c-Jun plus c-Fos AP-1 activity (approximately 5 fold). However, the presence of endogenous AP-1 proteins might complicate interpretation of these results. Therefore the Gal4 DNA binding domain was fused to the c-Jun or c-Fos transactivation domains and cotransfected these chimeric cDNAs with KRC and a Gal4 binding site-luciferase reporter construct into 293T cells. The chimeric GAL4-c-Jun, but not GAL4-c-Fos, protein potently transactivated the reporter construct in the presence of KRC demonstrating that KRC indeed acts as a transcriptional coactivator. In sum then, KRC specifically associates with c-Jun under physiologic conditions and this association augments AP-1 transcriptional activity.

EXAMPLE 22: KRC PHYSICALLY ASSOCIATES WITH c-Jun BUT NOT c-Fos

In this example, the results demonstrate that KRC physically interacts with c-Jun but not c-Fos. Expression vectors encoding c-Jun, c-Fos and a truncated myc-tagged version of KRC encoding amino acids 204 to 1055 (KRC tr) which includes the third zinc finger domain, one of the three acidic domains and the putative NLS sequence were overexpressed in the 293T kidney epithelial cell line. Coimmunoprecipitation using a monoclonal anti-myc antibody revealed that KRC physically associated with the c-Jun/c-Fos AP-1 complex. Further, it demonstrated that the region of KRC, aa 204-1055 shown to associate with TRAF2 also interacted with AP-1. KRC appeared to interact

with both members of the AP-1 complex. However, 293T cells express endogenous c-Jun. To test definitively whether KRC interacted with both members of AP-1, in vitro translated c-Fos, c-Jun and KRCtr were coimmunoprecipitated using antibodies to c-Jun, c-Fos and KRC. In this assay KRCtr interacted with c-Jun but not c-Fos. Further, the interaction between KRCtr and c-Jun required only the c-Jun N-terminal portion AA 1-79, termed the delta domain. It was possible that posttranslational modification of c-Fos was required for its interaction with KRC. Alternatively, KRC might interact with c-Fos only when it was associated with c-Jun. Indeed, when c-Jun was present in the lysates, c-Fos coimmunoprecipitated with KRCtr. These experiments revealed that KRC physically associated with c-Jun, but not c-Fos, the high affinity association of c-Fos with endogenous c-Jun presumably leading to the coimmunoprecipitation of c-Fos with KRC observed above. Consistent with this result was the failure to detect association of KRC with c-Fos in a yeast two hybrid assay .

EXAMPLE 23: KRC REGULATES THE STABILITY OF THE c-Jun/c-Fos AP-1 TRANSCRIPTION FACTOR THROUGH CONTROLLING ITS DEGRADATION

In this example, the results demonstrate that KRC regulates the stability of the c-Jun/c-Fos AP-1 transcription factor by controlling its degradation. The above experiments mapped the interaction site of KRC with c-Jun to aa 204-1055 of KRC. The interaction of full-length KRC with c-Jun was tested. However, attempts to demonstrate that full-length KRC interacted with AP-1 in overexpression experiments resulted in coimmunoprecipitation of very small amounts of c-Jun and no detectable c-Fos protein when compared to truncated KRC. These results raised the possibility that association of full-length KRC protein with AP-1 might lead to its degradation. Time course experiments were performed in which overexpressed sense KRC or an antisense KRC previously shown to block production of endogenous KRC protein were coimmunoprecipitated with overexpressed c-Jun and c-Fos. Overexpression of full-length KRC, in the presence of low dose cycloheximide to block endogeneous protein synthesis led to the rapid degradation of c-Jun. Conversely, overexpression of antisense KRC, by inhibiting the expression of endogenous KRC, decreased the rate of c-Jun degradation. The same set of experiments were performed using c-Fos, a very short-lived cellular protein. As with c-Jun, the stability of the c-Fos protein in the presence of

cycloheximide was compromised in the presence of KRC and dramatically stabilized in the presence of the KRC dominant negative expressing only the ZAS2 domain or in the presence of the antisense KRC. Remarkably, degradation of c-Fos was almost completely abolished in the presence of antisense KRC, suggesting that KRC may be the major protein that controls c-Fos degradation in vivo. The ability of KRC to promote the degradation of other fos family members Fra1, Fra2 and Fos B was also tested. Only c-Fos protein stability was deceased in the presence of KRC demonstrating the specificity of KRC for the c-Jun/c-Fos AP-1 pair. Viral Fos, an oncogene in acutely transforming retroviruses, contains a frameshift mutation that replaces the last 48 amino acids of c-Fos with an unrelated 49 amino acid-long C terminal tail that renders v-Fos a more stable protein compared to c-Fos. The increased stability accounts in part for the superior transformation ability of v-Fos. The protein stability of V-fos was not affected by altering levels of KRC by sense or antisense overexpression.

EXAMPLE 24: KRC REGULATES THE STABILITY OF THE c-Jun AND c-Fos BASED ON THEIR FUNCTION AS TRANSCRIPTIONAL ACTIVATORS

In this example, the results demonstrate that the effect of KRC in regulating the stability of c-Jun and c-Fos proteins is reflected in their ability to function as transcriptional activators. To examine the functional consequences of AP-1 degradation by KRC, cotransfection experiments in 293T cells with sense or antisense KRC together with a luciferase-tagged AP-1 reporter construct were performed. Overexpression of sense KRC resulted in decreased stimulation of AP-1 activity while conversely, expression of antisense or DN KRC led to an increase in AP-1 activity. To determine whether KRC alters both the level of activation per cell and the number of cells in which activation or repression occurs we used an AP-1 target site construct fused to GFP. Cotransfection of the AP-1-GFP construct together with KRC or antisense KRC into 293 cells revealed that KRC reduced both the number of cells in which GFP was expressed as well as the intensity of GFP expression per cell. Conversely, cotransfection of antisense KRC increased AP-1 transactivation as evidenced by an increased number of GFP+ cells as well as an increase in the intensity of fluorescence per cell in. Thus, the effect of KRC in regulating the stability of the c-Jun and c-Fos proteins is reflected in their ability to function as transcriptional activators.

EXAMPLE 25: KRC IS REQUIRED FOR UBIQUINATION OF

BOTH c-Jun AND c-Fos

In this example, the results demonstrate that KRC is required for ubiquitination of both c-Jun and c-Fos. Much attention has recently been focused on the role of covalent modification in controlling gene transcription in eukaryotes. Lysine modification by ubiquitination, sumoylation and acetylation of transcription factors contributes to their function in modulating gene expression. Previous studies have established that AP-1 proteins are rapidly degraded by the ubiquitin/ proteasome pathway. In this pathway, ubiquitin (UB) a 76 amino acid polypeptide is activated by the formation of a thiol ester linkage by the ubiquitin activating enzyme (E1) and is then transferred to the active site cysteine of a ubiquitin carrier protein (E2). Formation of an isopeptide bond between the C terminus of UB and lysines on a substrate is catalyzed by a UB ligase (E3), which binds the substrate and catalyzes the transfer of the UB from a specific E2 to the substrate. The formation of a chain of UB molecules on the substrate then targets it for degradation by the 26 S proteasome. It has been shown that KRC interacts with AP-1 to regulate its degradation raising the possibility that KRC might be the elusive AP-1 E3 UB ligase responsible for its ubiquitination in vivo.

EXAMPLE 26: KRC KNOCKOUT B CELLS HAVE IMPAIRED IgA PRODUCTION AND TGFβ-DEPENDENT GLα TRANSCRIPTION

Homozygous mutant KRC KO mice have normal lymphocyte development as determined by FACS analysis of primary and secondary lymphoid organs. Despite normal B cell development, analysis of serum immunoglobulins (Igs) in non-immunized KRC KO mice revealed a selective reduction of circulating IgA. The decrease in serum IgA correlated with observations *in vitro* that purified splenic CD 19+ B cells from KRC KO mice, activated under conditions that promote IgA class switching, secreted significantly lower levels of IgA than WT B cells.

To determine if KRC regulates IgA production at the level of transcription, Iga germline transcripts (GLα) in activated WT and KRC KO B cells were analyzed. Consistent with the decreased IgA secretion observed, activated KRC KO B cells had a marked reduction in levels of GLα transcripts when compared to WT B cells. It has previously been reported that TGFβ signaling in B cells has a central role in regulating GLα transcription through SMAD3 and Runx3 mediated processes (Zhang, Y., and

Derynck, R. 2000. J Biol Chem 275: 16979-16985; Shi, M. J., and Stavnezer, J. 1998. J Immunol 161: 6751-6760.).

Given the findings that KRC can interact with SMAD3, it was determined whether KRC could augment the transcriptional activity of SMAD3 and/or Runx3 in driving the expression of GLα. A luciferase reporter plasmid driven by the mouse GLα promoter (-179/+46) was cotransfected into the M12 B-cell line along with KRC, Runx3 and/or SMAD3 expression constructs. Cotransfection of KRC enhanced the ability of SMAD3 and Runx3 to drive the expression of the reporter plasmid ~~(Figure 23D)~~. M12 cells express endogenous SMAD3 and therefore it is not clear if the effects of KRC on Runx3 may be independent of SMAD3. Therefore KRC regulates IgA class switching as well as other B cell effector functions by acting downstream of the TGFβ receptor in these cells.

Signaling by Decapentaplegic (Dpp), a member of the TGFß superfamily of signaling molecules similar to vertebrate BMP2 and BMP4, has been implicated in many developmental processes in Drosophila melanogaster. Notably, Dpp acts as a long-range morphogen during imaginal disc growth and patterning. Genetic approaches led to the identification of a number of gene products that constitute the core signaling pathway. Decapentaplegic (Dpp) signaling leads to association of Medea (Med) with Mothers against *dpp* (Mad) Mammalian homologues of the Drosophila Med and Mad proteins are the SMADs. Once Dpp associates with Med and Mad, it then translocates to the nucleus where it interacts with Schnurri. In addition to Schnurri, Dpp signaling and Brinker (Brk), to prime cells for Dpp responsiveness.

It has been demonstrated that Schnurri is required for Dpp-mediated gene repression. It was therefore determined whether KRC could interact with the mammalian homologue of Mad, SMAD3. KRC physically interacts with two R-SMADs, SMAD3 and to a lesser extent with SMAD2 but does not interact with the Co-SMAD, SMAD4. This is consistent with what has been observed in Drosophila, where Shn interacts with Mad but not Med. In addition, it was found that KRC enhances the transcriptional ability of SMAD3 to drive expression of a luciferase reporter construct containing a basic SMAD-binding element.

EXAMPLE 27: KRC AUGMENTS Th2 CYTOKINE PRODUCTION AND INTERACTS WITH GATA3

Composite AP-1/NFAT sites are found in the proximal promoter regions of many cytokine genes such as TNFα, GM-CSF, IL-2, IL-3, IL-4, and IL-5 (Rao, A. 1994. Immunology Today J_5: 274-281; Rooney, J. et al. 1995. Immunity 2: 473-483.). Given that KRC is an inducible AP-1 coactivator for the IL-2 gene, it was determined whether KRC could regulate other AP-1 -dependent genes in T cells. A systematic analysis of cytokine production by primary lymph node (LN) and splenic CD4+ T cells stimulated by plate-bound anti-CD3/CD28 in the absence of polarizing cytokines (unskewed conditions) from KRC WT and KO mice was performed. As was previously published, KRC KO CD4+ cells showed a striking defect in IL-2 production at early time points (up to 36 hours) (Oukka, M., et al. 2004. J Exp Med 199: 15-24.). Additionally, KRC KO T cells displayed reduced proliferation at early time points compared to WT cells, as measured by ^{3}H incorporation. This proliferation defect was completely rescued by the provision of exogenous hIL-2, indicating that it was due completely to reduced IL-2 production. Moreover, analysis of IL-2 production by real-time PCR and ELISA at later time points of primary stimulation showed that KRC KO T cells produced levels of IL-2 equivalent to WT cells at all time points during primary anti-CD3/CD28 stimulation beyond 36 hours showing redundancy by other Schnurri family members. LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 μg/ml) for 72 hours in the presence of 200 U/ml human IL-2. Supematants were analyzed for IFNy, IL-4, and IL-5 levels by ELISA. For subsequent experiments described below, exogenous hIL-2 was added to all cultures to account for any early differences between WT and KRC KO CD4+ T cells. Analysis of Th effector cytokine production revealed dramatic differences between KRC WT and KO cells following 72 hours of primary unskewed stimulation. While KRC WT and KO cells secreted similar levels of the Thl effector cytokine IFNγ, production of Th2 effector cytokines IL-4 and IL-5 was drastically reduced in KRC KO cells despite normal proliferation, indicating that the defective Th2 cytokine production was not due to decreased cell division.

To investigate the consequences of decreased IL-4 and IL-5 in these unskewed primary stimulations, LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 μg/ml) for 72 hours in the presence of 200 U/ml human IL-2 (unskewed). Cells were expanded for an

additional 4 days in the presence of hIL-2 and restimulated with plate-bound anti-CD3. As expected, production of all Th2 effector cytokines was dramatically reduced in secondary stimulations of KRC KO cells. However, when cells were initially stimulated under Th2-polarizing cytokines (IL-4 plus neutralizing antibodies to IFNγ), production of Th2 effector cytokines by KRC KO cells was identical to WT cells. LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 ug/ml) for 72 hours in the presence of hlL-2, IL-4 and neutralizing antibodies to IFNγ (Th2-skewed). Cells were expanded for 3 days in hlL-2, and restimulated for 18 hours with plate-bound anti-CD3 (2 μg/ml). Supematants were analyzed for IL-4, IL-5, IL-6, IL-10, and IL-13 levels by ELISA. These results indicated that KRC KO cells were not defective *per se* in producing Th2 cytokines; rather, KRC was required for the establishment of the Th2 effector cell under unskewed primary stimulation conditions. Given that KRC mRNA is rapidly induced in Thp cells following TCR/CD28 ligation (Oukka, M., et al. 2004. J Exp Med 199: 15-24) and that KRC mRNA levels fall 2-3 days following primary T cell activation, it shows that KRC induction plays a role in reinforcing the activity of factors required for Th2 cell generation. Moreover, since KRC KO cells produce perfectly normal levels of IL-2 at later time points, and KRC KO Th2 effector cells secrete normal levels of all AP-1-dependent Th2 cytokines, these results strongly suggested that KRC's role in Th2 cell generation was independent from its ability to function as an AP-1 coactivator.

To further analyze the defect in Th2 cell generation in the absence of KRC, RNA and cDNA were prepared from WT and KRC KO CD4+ T cells at 0, 12, 24, and 48 hours following anti-CD3/CD28 stimulation in unskewed conditions. LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 μg/ml) for the indicated times in the presence of 200 U/ml human IL-2. RNA and cDNA were made and analyzed for the presence of IL-4 and GATA3 mRNA relative to fi-actin using real-time PCR. Levels of IL-4 and GATA3 transcripts were analyzed by real time PCR. Although initial induction of IL-4 mRNA was comparable between WT and KO cells, KRC KO cells were unable to fully upregulate IL-4 following 48 hours of CD3/CD28 stimulation. Strikingly, this defect in production of high levels of IL-4 mRNA was accompanied by nearly absent upregulation of the Th2-specific transcription factor GATA3 at these time points.

Since the defect in GATA3 upregulation preceded the defect in IL-4 upregulation, the primary lesion in Th2 cell generation in the absence of KRC was in the early induction of GAT A3. Therefore, WT and KRC KO cells were transduced with control GFP and bicistronic GFP-GATA3 retroviruses 24 hours following primary TCR/CD28 stimulation in unskewed conditions. Cells were then expanded in hIL-2, restimulated with PMA/ionomycin, and assayed for secondary Th2 cytokine production by intracellular cytokine staining. LN and splenic CD4+ cells from KRC WT and KO mice were stimulated by plate-bound antibodies to CD3 (2 μg/ml) and CD28 (1 μg/ml) for 24 hours in the presence of hIL-2. Cells were then infected with retroviruses expressing either GFP or GFP-GATA3. Cells were expanded in hIL-2 for 3 days, and subsequently restimulated with PMA/ionomycin for 6 hours. Intracellular cytokine staining to analyze IL-4 production in GATA3-negative and GATA3-positive cells was performed. As expected, GFP-negative and control GFP-expressing KRC KO cells showed reduction in intensity of IL-4 and absolute cell number of IL-4 producers ~~(Figure 24G)~~. Additionally, although levels of GFP were identical between WT and KO RV-GATA3 cultures, KRC KO RV-GATA3 -expressing cells failed to express levels of IL-4 comparable to WT RV-GATA3-expressing cells. These results indicated that KRC lay both upstream and downstream of GATA3 in its ability to regulate the generation of IL-4-producing Th2 cells.

In addition to its ability to directly transactivate the IL-5 and IL-13 genes and to induce chromatin remodeling of the entire Th2 cytokine locus, another well-documented property of GATA3, like many 'master regulator' transcription factors, is its ability to auto-activate itself (Ouyang, W., et al. 2000. Immunity 12: 27-37). Since both GATA3 induction and GATA3 activity were reduced in KRC KO cells, KRC plays a role in directly regulating the function of GATA3, in its ability to auto-activate itself and/or in its ability to drive activation of the Th2 cytokine locus. Since KRC can interact with SMAD3 and SMAD3 can bind and potentiate GATA3-driven transcription (Blokzijl, A., et al. 2002. Curr Biol 12: 35-45), KRC could regulate GATA3 activity by binding GATA3 itself. 293T cells were transfected with KRC with or without FLAG-GATA3. 48 hours later, cells were lysed and FLAG-tagged proteins were immunoprecipitated overnight with anti-FLAG beads. Immunoprecipitates were washed, resolved by SDS-PAGE, and KRC was detected by immunoblotting. When overexpressed in 293T cells,

FLAG-GATA3 specifically precipitated overexpressed KRC. To evaluate the function of this physical interaction, the ability of overexpressed KRC to regulate GATA3-driven transcription from an IL-5-luciferase construct (Miaw, S. C, et al. 2000. Immunity 12: 323-333) was tested in EL4 cells. While KRC had no effect on IL-5-driven transcription in the absence of co-expressed GATA3, the combination of KRC and GATA3 led to dramatic enhancement of GATA3 transcriptional activity, consistent with the previously-described role for KRC as a transcriptional coactivator. Note that neither Shn-1 nor Shn2 could augment GATA3-dependent IL-5 promoter activation. Finally, to determine whether KRC could potentiate GATA3-driven auto-activation of the GATA3 gene itself, the ability of KRC to potentiate GATA3's ability to drive activation of different segments of the GATA3 genomic locus fused to luciferase was tested (Hwang, E. S., et al. 2002. J Immunol 169: 248-253). Much like its ability to potentiate GATA3's activity on the IL-5 promoter, KRC also strongly enhanced the ability of GATA3 to drive expression from a previously described intronic enhancer between exons 1 and 2 of the GATA3 locus. EL4 cells were electroporated with 1 μg IL-5-luciferase reporter or 1 ug of GATA3-luciferase reporters with combinations of GATA3 (4 μg) and Shns 1,2 and 3 (20 μg). 18 hours later, cells were stimulated with PMA/ionomycin for 6 hours and luciferase activity was determined.

EXAMPLE 28: KRC DEGRADES ITS PARTNERS

In the course of mapping the interaction site of KRC with c-Jun, it ws observed that coimmunoprecipitation of full-length KRC with c-Jun in overexpression experiments resulted in very small amounts of c-Jun and no detectable c-Fos protein when compared to truncated KRC. These results raised the possibility that association of full-length KRC protein with its partners might lead to their degradation. Experiments in which KRC was coexpressed with c-Jun, c-fos, SMAD3, Runx2, GATA3 and TRAF2 were performed. 293T cells were transiently transfected with c-Jun, c-Fos, or FLAG-tagged Smad3, Runx2, Gata3, and Traf2 with or without KRC. 48 hours later, cells were treated with 10 ug/ml cycloheximide for 15 minutes. Whole cell lysates were prepared and 30 ug protein/sample was resolved by SDS-PAGE followed by immunoblotting for c-Jun, c-Fos, or FLAG. Blots were stripped and reprobed with anti-Hsp90 antibody as a loading control. Overexpression of full-length KRC in the presence of low dose cycloheximide to block endogeneous protein synthesis led to the rapid degradation of all

of these proteins. However, KRC augments cJun, SMAD3 and GATA3-dependent gene activation despite its ability to degrade these transcription factors. Ubiquitination of transcription factors leads to their degradation but also can increase their potency in transactivation simultaneously with their degradation (Molinari, E., et al. 1999 Embo J 18:6439-6447; Salghetti, S. E., et al. 2001. Science 293: 1651-1653; von der Lehr, et al. 2003. Mol Cell H: 1189-1200; Grossman, S. R., et al. 2003. Science 300: 342-344; Greer, S. F., et al. 2003. Nat Immunol 4: 1074-1082.). Further, inclusion of the proteasome inhibitor MG-132 prevented the degradation of Fos by KRC. Therefore, KRC might be an E3 ubiquitin ligase.

EXAMPLE 29: KRC UBIQUITINATES ITS PARTNERS, TRAF2 AND Runx2

It is known that KRC physically associates with the above transcription factors, and that this association results in the degradation of these proteins. One major pathway for protein degradation is the ubiquitin/protesasome complex. In preliminary ubiquitination assays, increased ubiquitination of two KRC partners, TRAF2 and Runx2, was detected, demonstrating that KRC functions as a component of an E3 ligase. These experiments were performed by transiently transfecting 293T cells with FLAG-tagged Runx2 or Traf2 with or without KRC. 48 hours later, cells were treated with 10 ug/ml cycloheximide for 15 minutes. Whole cell lysates were prepared and 30 ug protein/sample was resolved by SDS-PAGE followed by immunoblotting for FLAG. Blots were stripped and reprobed with anti-Hsp90 antibody as a loading control. 293T cells were transiently transfected with FLAG-tagged Runx2 or Traf2 with the indicated combinations of His-Ubiquitin and KRC. 48 hours later, cells were treated with the proteasome inhibitor MG132 (10 uM) for 2 hours. Cells were lysed in 6M guanidium-Hcl, and His-ubiquitin-conjugated proteins were precipitated with Ni-NTA agarose. Precipitates were washed and resolved by SDS-PAGE followed by immunoblotting anti-FLAG to detect poly-ubiquitinated Runx2 or Traf2 species.

The functional outcome of TRAF2 and Runx2 degradation is straightforward since KRC actually represses TRAF2 and Runx 2 driven responses in vitro (Oukka, M., Kim, et al. Mol Cell 9: 121-131).

EXAMPLE 30: Shn2 and KRC HAVE OVERLAPPING BUT UNIQUE FUNCTIONS

Mice that lack Shn2 have severely impaired positive selection of CD4+ and CD8+ cells, and peripheral CD4 T cells had impaired production of IL-2 (Takagi, T., et al. 2001. Nat Immunol 2: 1048-1053). The mechanism by which Shn2 acts to control these functions has not been established. In order to determine how Shn2 controls these T cell functions, Jurkat cells were electroporated with 2 ug 2xAP-1-luciferase reporter along with 20 ug vector, Shn2, or KRC DNA. Eighteen hours later, cells were stimulated with PMA/ionomycin for 6 hours and luciferase activity was determined. Like KRC, Shn2 associates with AP-1 to transactivate an AP-1 reporter. However, Shn2 does not coactivate SMAD3 or GATA3's ability to transactivate the GLα or IL-5 genes, respectively, in the absence or presence of TGFβ.

EXAMPLE 31: PHENOTYPIC ANALYSIS OF KRC KNOCKOUT ANIMALS

The most pronounced immune system abnormalities of these mice are evidence of impaired TGFβR signaling in B cells and impaired early development of the T helper 2 (Th2) lineage from its progenitor (Thp), i.e. KRC KO Th cells have impaired production of Th2, but not Th1 cytokines. Analysis of serum Igs in KRC KO mice as well as *in vitro* secretion of Igs by KRC KO B cells has also revealed a role for KRC in the regulation of IgA production.

The generation and regulation of effector B cell functions involve a complex temporal network of cytokines, signaling proteins, and transcription factors. Dysregulation of any one component may compromise the B cell's ability to mediate its effector functions and contribute to a failure of the host immune system to effectively respond to foreign pathogens. As described above, deletion of KRC results in impaired IgA secretion and transcription of the GLα gene in vivo.

TGFβ has been demonstrated to influence various aspects of normal B cell biology and is important in regulating humoral immune responses. In normal cells, TGFβ signaling is initiated when this molecule binds to and induces a heterodimeric cell-surface complex consisting of type I (TbRI) and type II (TbRII) serine/threonine kinase receptors. This heterodimeric receptor then propagates the signal through phosphorylation of downstream target SMAD proteins. There are three functional classes of SMAD protein, receptor-regulated SMADs (R-SMADs), Co-mediator SMADs (Co-SMADs) and inhibitory SMADs (I-SMADs). Following phosphorylation by the heterodimeric receptor complex, the R-SMADs complex with the Co-SMAD and

translocate to the nucleus, where in conjunction with other nuclear proteins, they regulate the transcription of target genes (Derynck, R., et al. (1998) Cell 95: 737-740).

Mice with a B-cell specific inactivation of TβRII have increased B-cell responsiveness, enhanced antibody production and a selective defect in the production of antigen-specific IgA (Cazac, B. B., and Roes, J. (2000) Immunity 13: 443-451). Further analysis of TGFβ signaling in B cells has demonstrated that this cytokine can modulate the expression of approximately 100 different genes in B cells (Roes, J., et al. (2003) Proc Natl Acad Sci U S A 100: 7241-7246). TGFβ can elicit different cellular responses in B cells through its ability to positively and negatively regulate gene transcription. Both activation and repression of gene expression by TGFβ utilize the same set of ubiquitous SMAD proteins. However, specific cofactors that bind to SMADs are believed to dictate whether a gene is upregulated or downregulated in response to TGFβ (Shi, Y., and Massague, J. (2003) Cell 113: 685-700). A similar transcriptional mechanism may account for the variable effects of TGFβ on B-cell effector function. Identification of the different cofactors expressed in B cells will be critical to fully understand how TGFβ regulates B cell function.

Disruption of those molecular pathways that regulate B cell function may also contribute to the development of B-cell leukemia and lymphomas. Most lymphoid neoplasms have chromosomal translocations or mutations that allow them to bypass the normal cellular checkpoints that control their propagation. During normal physiological processes, TGFβ serves as a potent negative regulator of cell growth and differentiation, thus serving as a key tumor suppressor. Several hematopoietic neoplasms, including B cell chronic lymphocytic leukemia (B-CLL), have genetic alterations that impair TGFβ signaling in these cells and render them nonresponsive to the growth-inhibiting effects of TGFβ (Schiemann, W. P., et al. (2004) Cancer Detect Prev 28: 57-64).

No role for the mammalian Shn genes in TGFβ signaling has yet to be identified although the three known vertebrate Shn orthologs have been postulated to be downstream of the bone morphogenetic protein-transforming growth factor-beta-activin signaling pathways (Rusten, T. E., et al. (2002) Development 129: 3575-3584). Given the well-defined role of Drosophila Shn in regulating Dpp, it was determined whether KRC is a component of the TGFβ signaling pathway. Indeed, it has been demonstrated that KRC physically interacts with two R-SMADs, SMAD3 and to a lesser extent with

SMAD2, but does not interact with the Co-SMAD, SMAD4. This is consistent with what has been observed in Drosophila, where Shn interacts with Mad but not Med. In addition, KRC enhances the transcriptional ability of SMAD3 to drive expression of a luciferase reporter construct containing a basic SMAD-binding element.

KRC is not downstream of TGFβR in T cells but that it is downstream of the TGFβR in osteoblasts as well as in B cells. A profound abnormality in development of the skeletal system is present in KRC KO mice. These mice exhibit an osteosclerotic phenotype that is characterized by increases in trabecular bone mass, bone mineral density and bone formation consistent with impaired signaling through the TGFβ receptor. While SMAD3 and the transcription factor Runx3 interact to activate transcription of the GLα gene, SMAD3 and another Runx family member, Runx2 act to repress transcription of the osteocalcin gene. KRC interacts with all three transcription factors. However, while KRC is a coactivator of GLα promoter activity, it is a corepressor of the osteocalcin gene. Hence, in its absence, GLα transcription is diminished in B cells but osteocalcin gene transcription is augmented in osteoblasts.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.